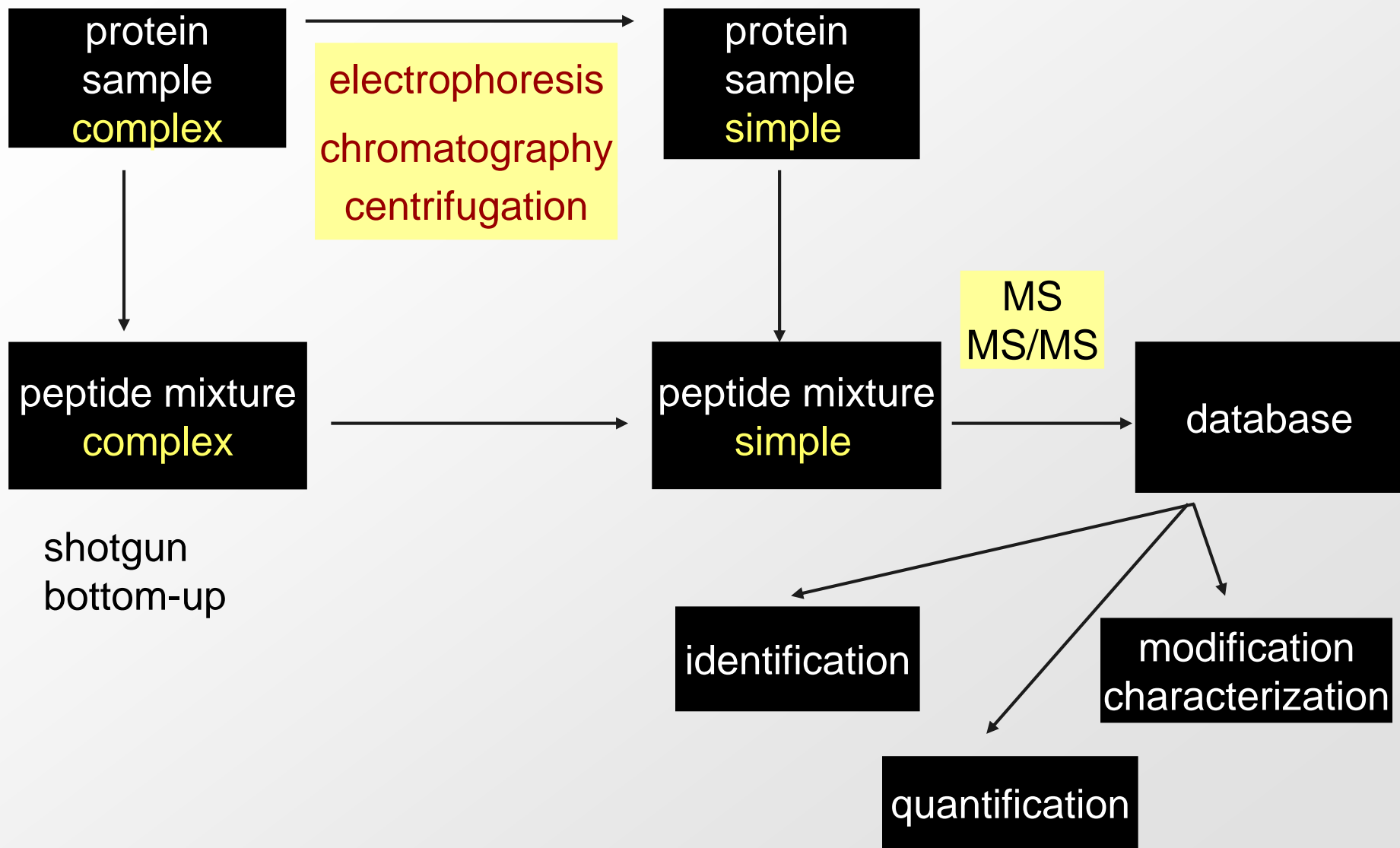


PROTEOMIC SAMPLE PREPARATION

Two-dimensional electrophoresis



Hana Konečná
Proteomics Core Facility
CEITEC Central European Institute of Technology
NCBR National Centre for Biomolecular Research

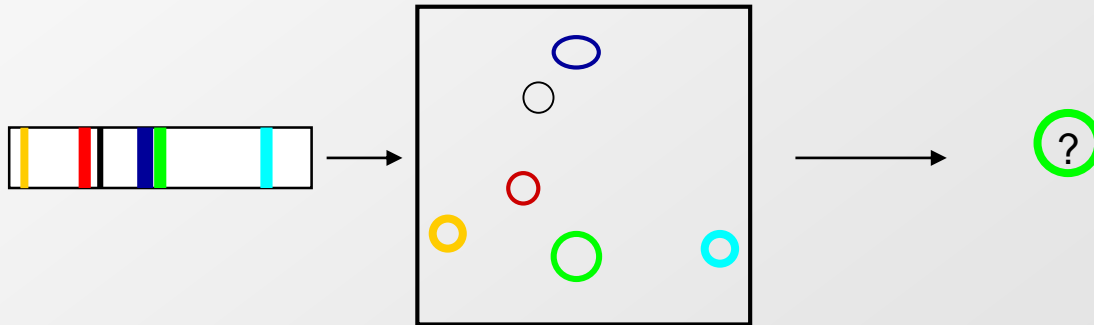


shotgun
bottom-up

I. SEPARATION

II. PREFRACTIONATION

Two-dimensional electrophoresis 2-DE



Proteomic experiment

2DE

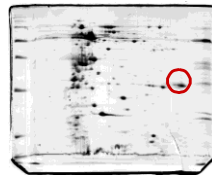
extraction



focusing



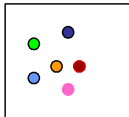
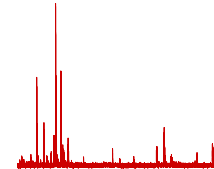
SDS-PAGE



digest



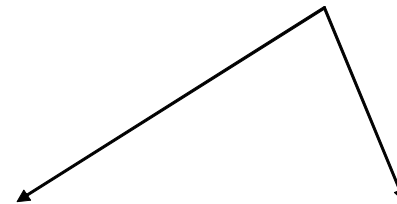
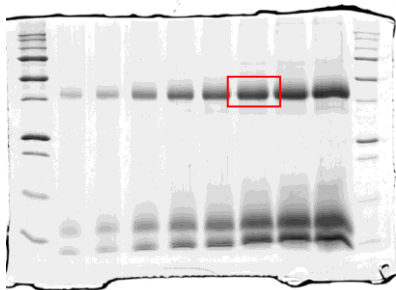
MS



identification

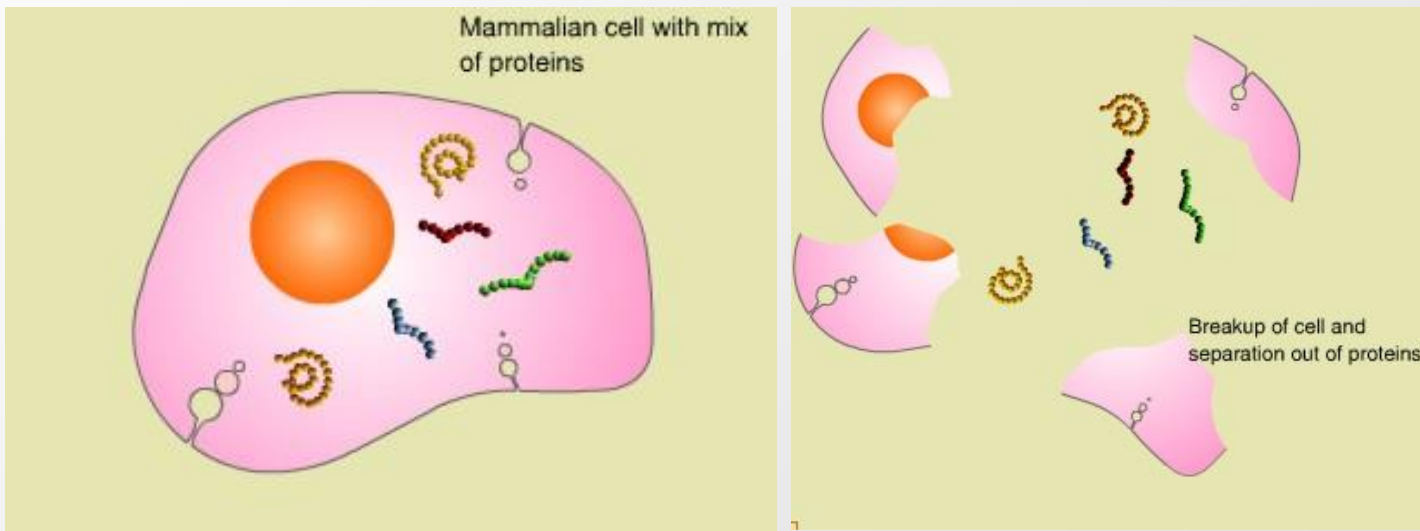
unknown protein

1DE



HOMOGENIZATION

- mechanical
- ultrasound
- pressure
- freeze/thaw lysis
- detergent lysis





CryoMill

Liquid Nitrogene



Watch for keratins!

SAMPLE PREPARATION

Solubilization urea thiourea detergents

Reduction

DTT

TBP

TCEP

dithiotreitol

tributylphospine

Tris (2-carboxyethyl)
phosphine hydrochloride

Inhibition

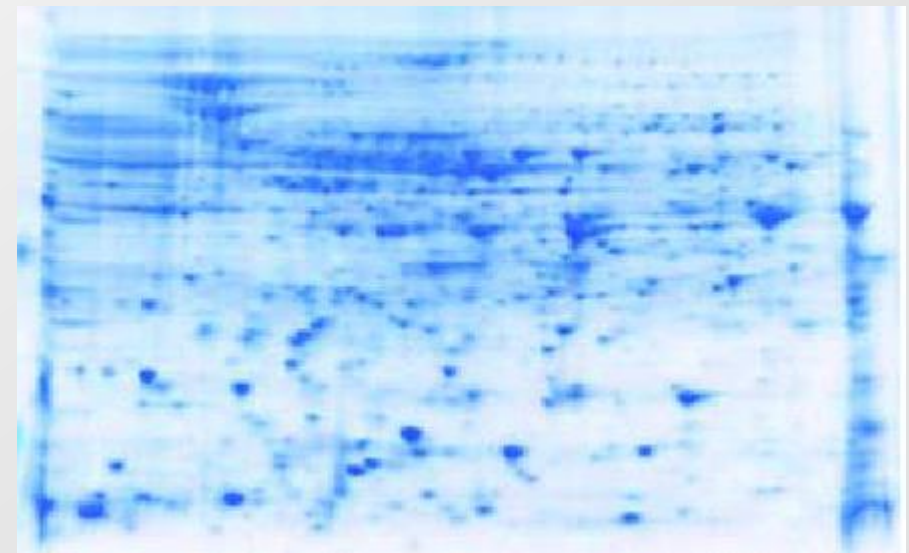
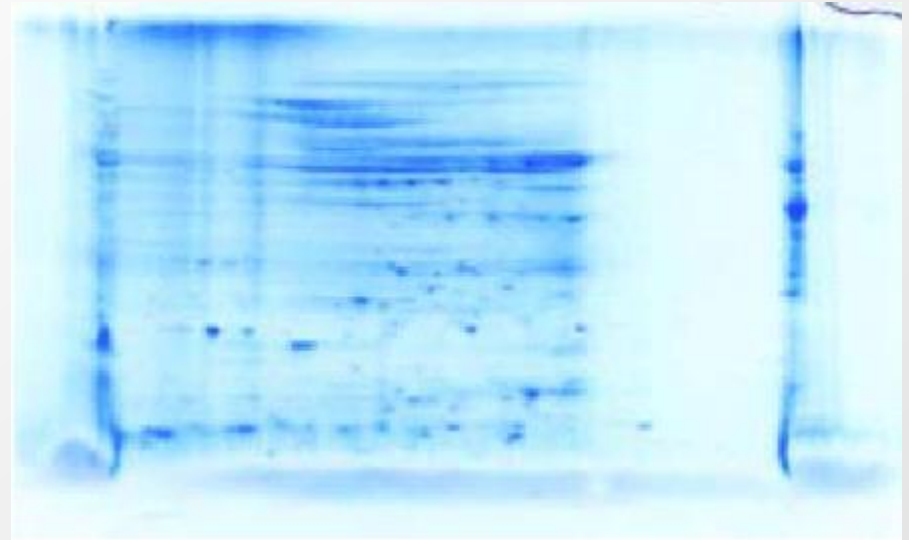
of proteases, phosphatases glycosylases

Contaminants removal

Protein assay

DETERGENTS

- no net charge
- 0.5 – 4%
- working in high urea
- non ionogenic
- zwitterion
- SDS only up to 0.25%

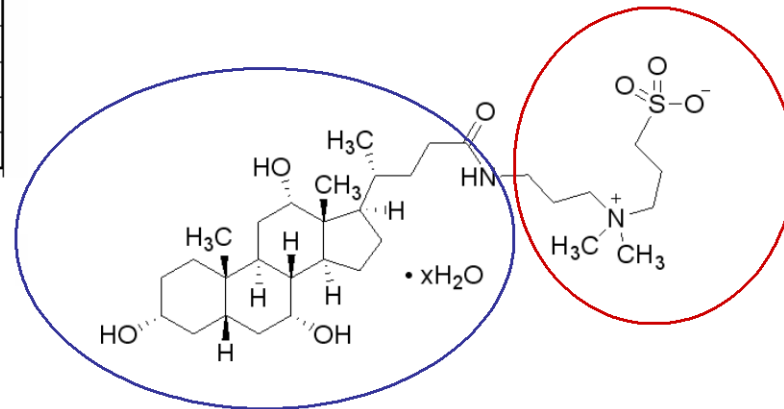
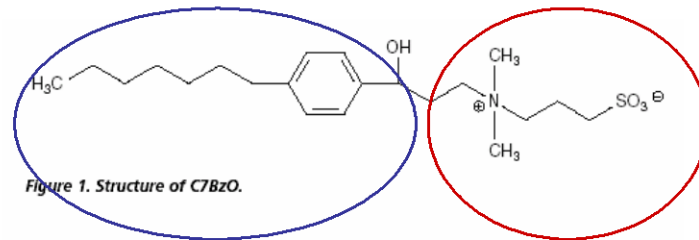
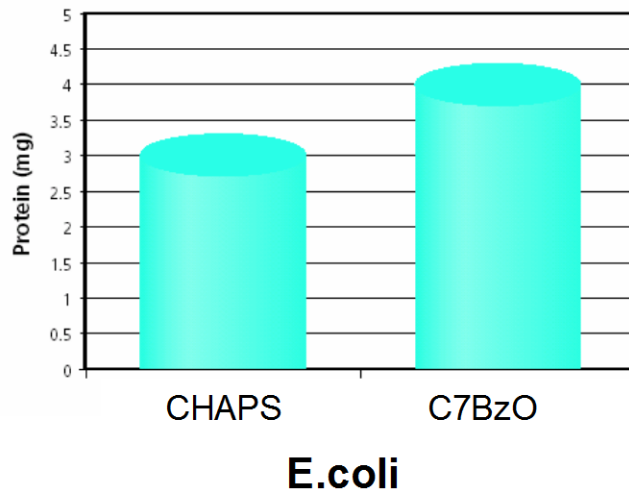


C7BzO

3-(4-Heptyl)phenyl-3-hydroxypropyl)dimethylammonio)propanesulfonate

CHAPS

3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate



RULE OF THUMB

- avoid proteolysis
- simple preparation
- fresh reagents
- fresh sample
- remove particles - spin
- remove contaminants

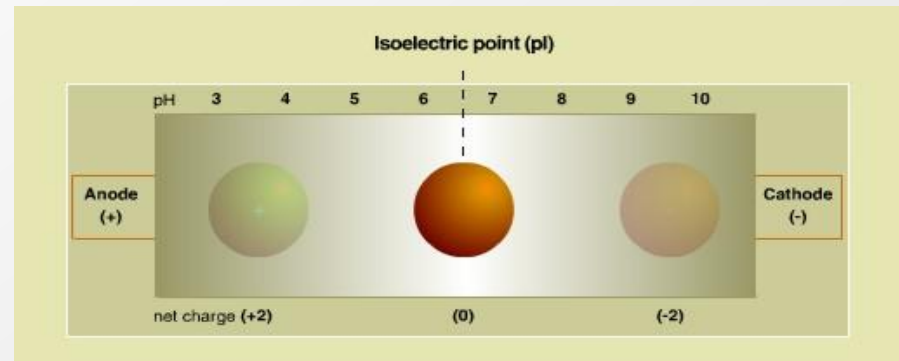
CONTAMINANTS

- salts, buffers
- small molecules
- ionic detergents
- nucleic acids
- polysaccharides
- lipids
- phenols

2-DE

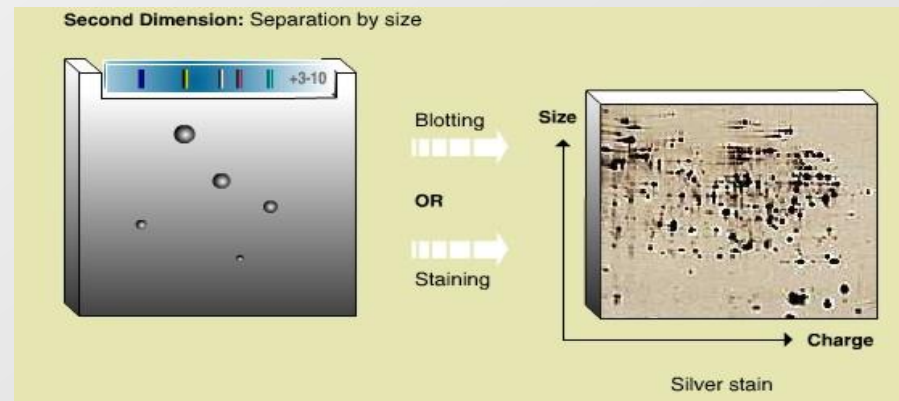
- first dimension

IEF



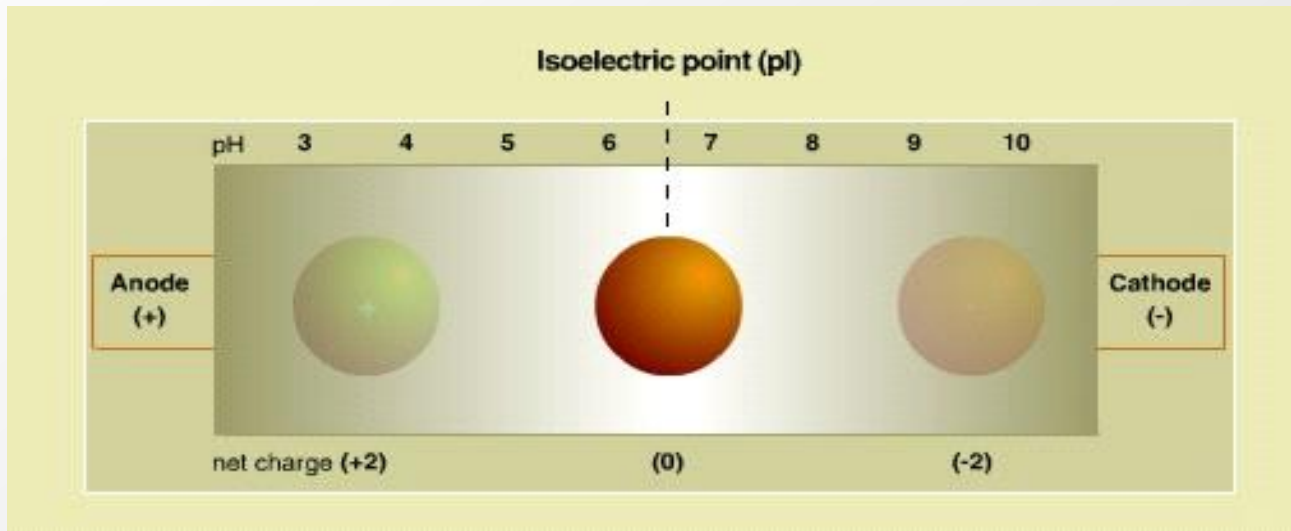
- second dimension

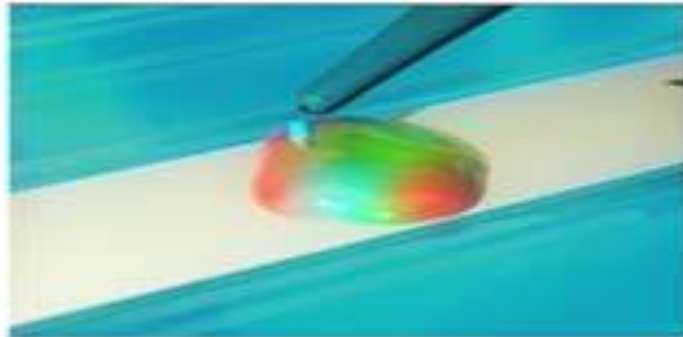
SDS-PAGE



1st dimension **ISOELECTRIC FOCUSING**

migration of charged molecules in pH gradient in electric field





-

ISOELECTRIC FOCUSING

- immobilized pH gradient
- ampholytes



-



-

+

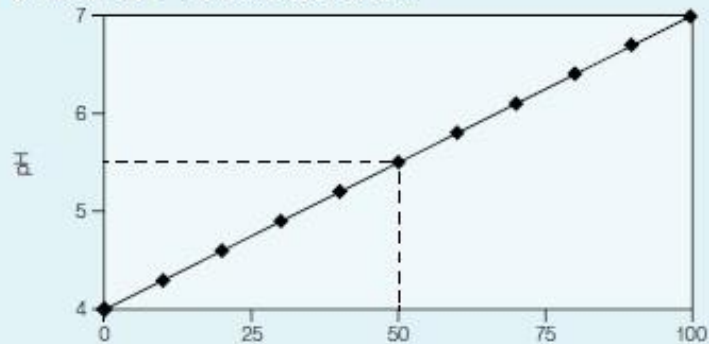
+

+

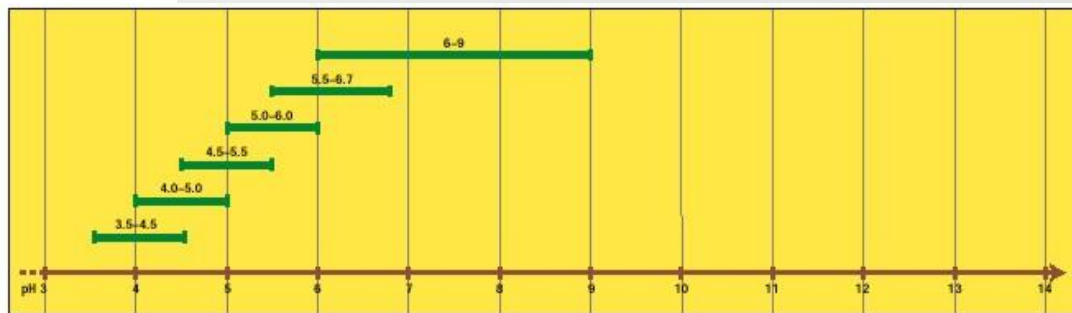
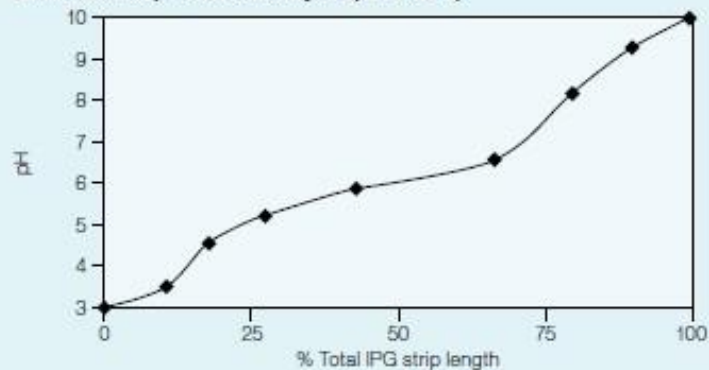
RANGE OF STRIP SIZE OF STRIP



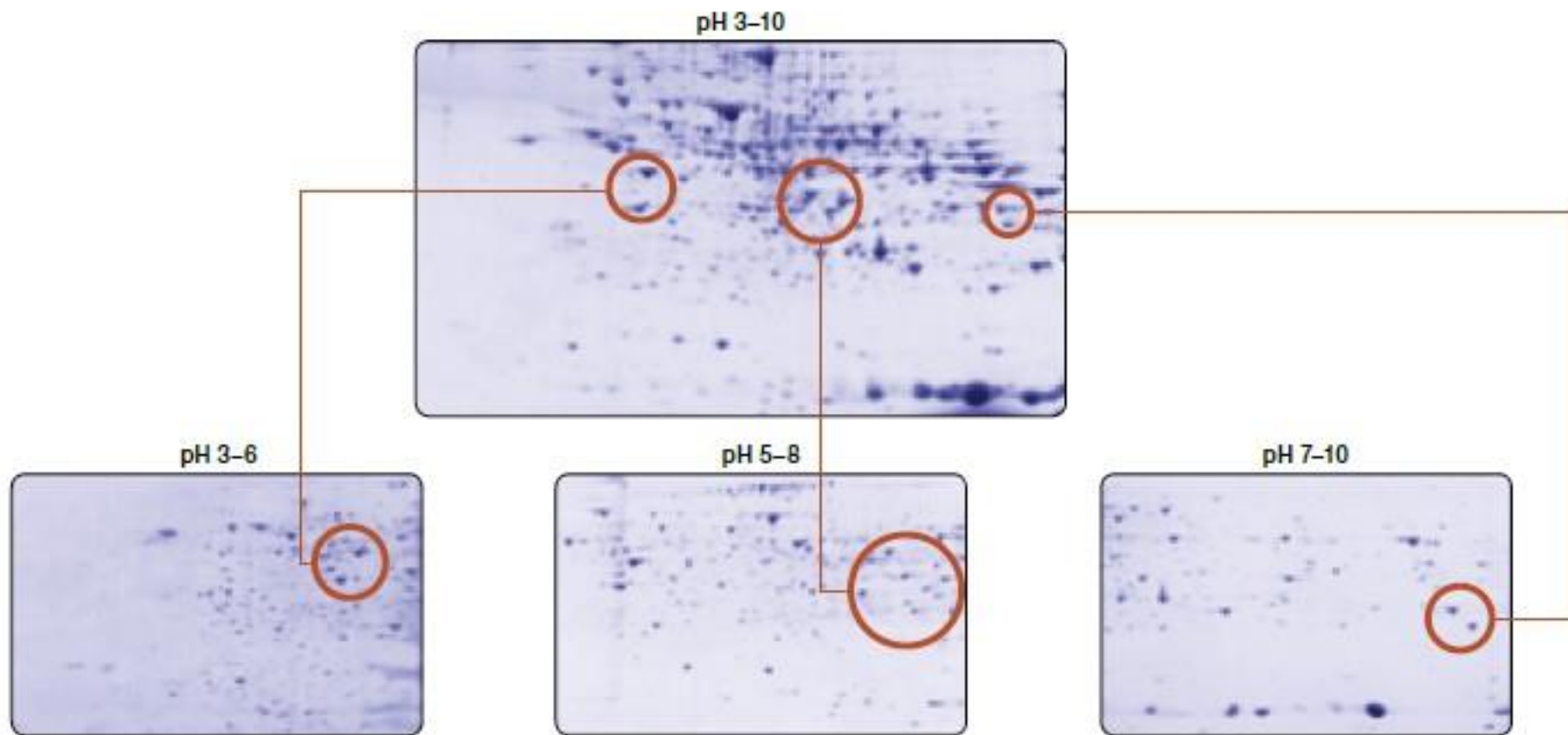
A. Linear pH 4-7 ReadyStrip IPG strip



B. Nonlinear pH 3-10 ReadyStrip IPG strip



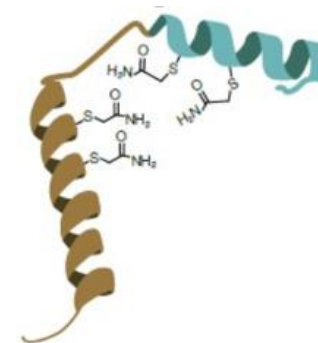
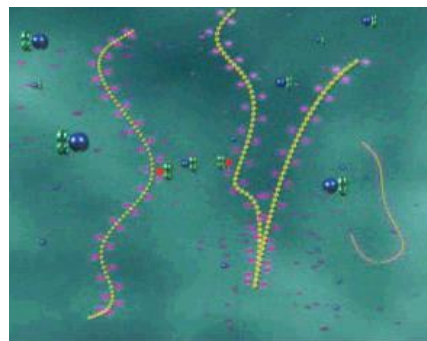
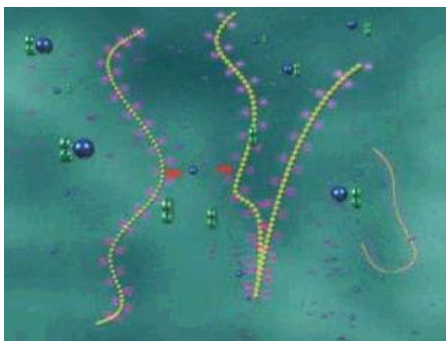
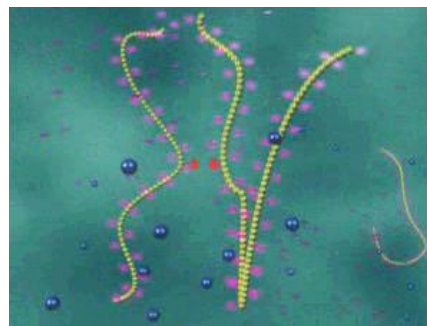
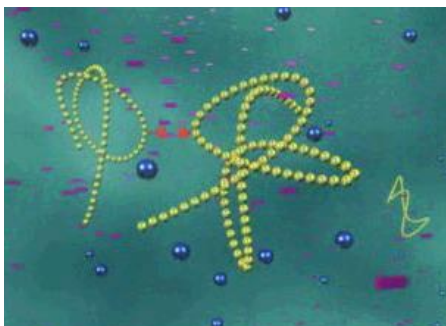
RANGE OF STRIP



EQUILIBRATION OF STRIP



denaturation **SDS** ●



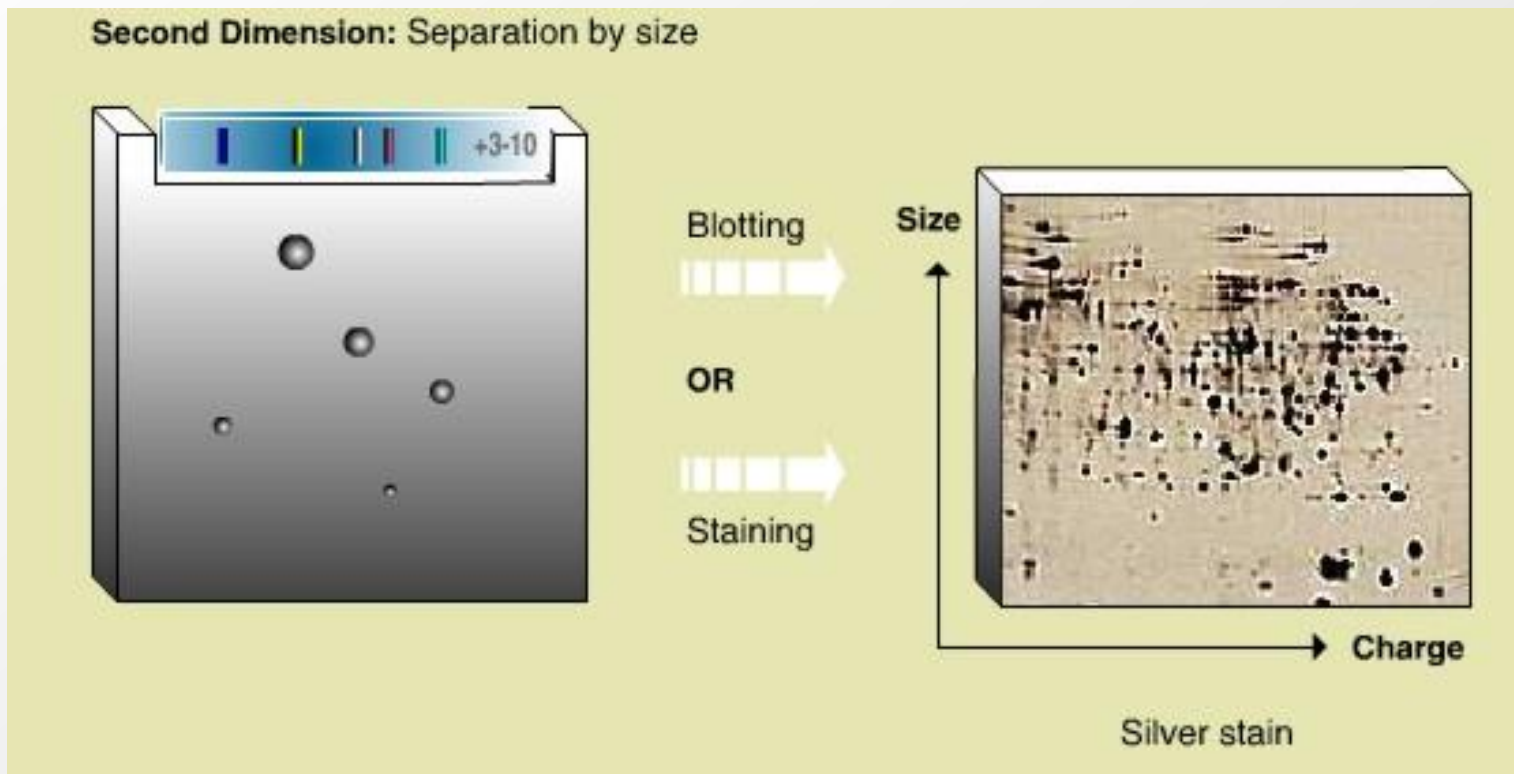
reduction **DTT** ●

alkylation **IAA** ●

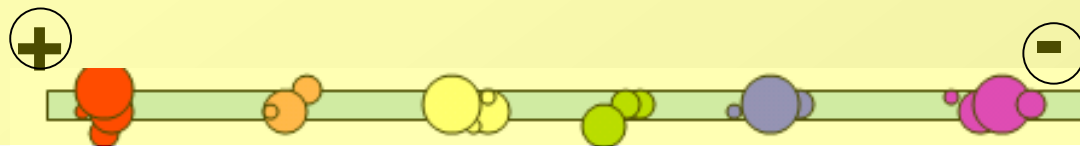
2nd dimension SDS-PAGE



Migration of anions in electric field according to MW



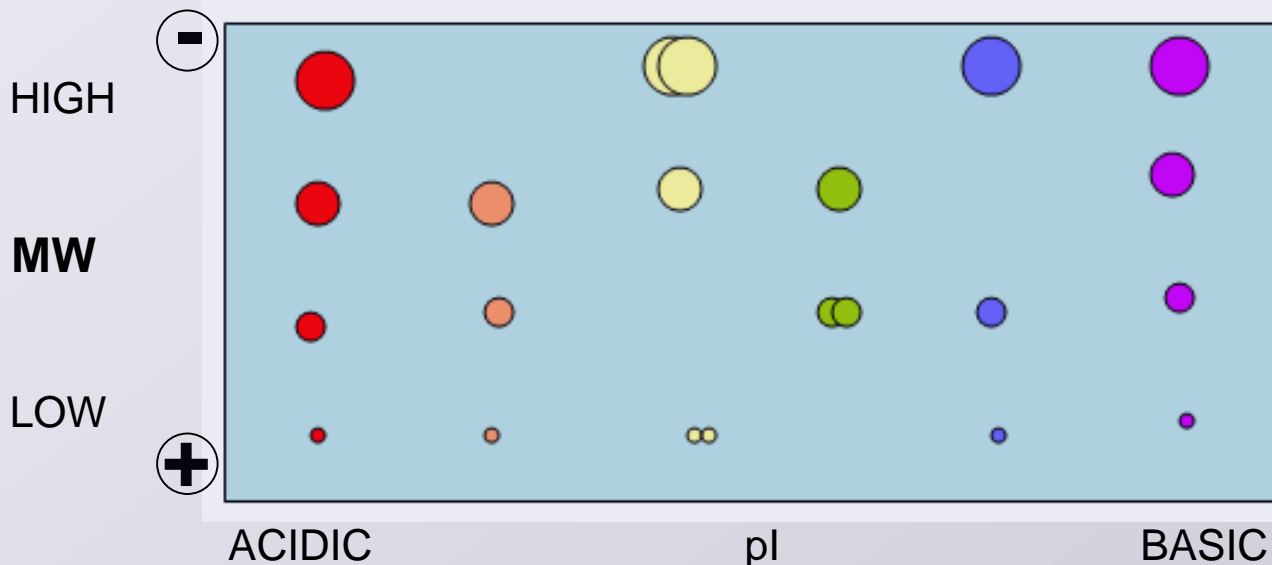
FOCUSING



STRIP

SDS-PAGE

↓ equilibration



GEL

Gel orientation

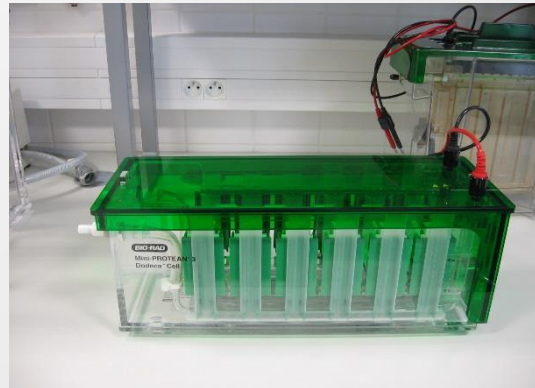


2-DE INSTRUMENTATION

- Protean IEF
 - Protean Dodeca Cell
 - Densitometer GS-800
 - FLA-7000, STORM
- PDQuest, Quantity One*



Protean Plus Dodeca Cell



Mini-Protean 3 Dodeca Cell



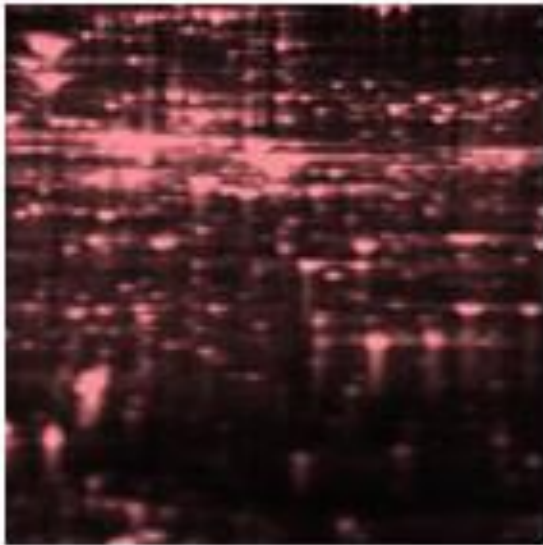
Protean II xi Cell



PROTEIN DETECTION

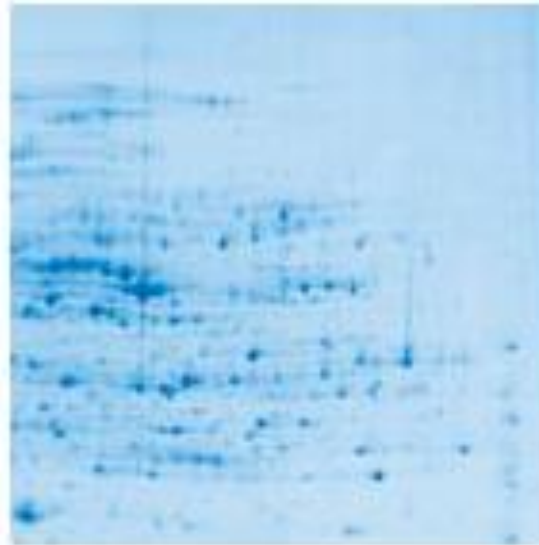
- gel x blot
- visualisation
 - staining
 - radioactivity assay
 - immunodetection
- staining in gel
 - post-electrophoretic
 - pre-electrophoretic
 - protein specific
 - PTM specific
 - visible spectrum
 - fluorescence

PROTEIN DETECTION IN GEL



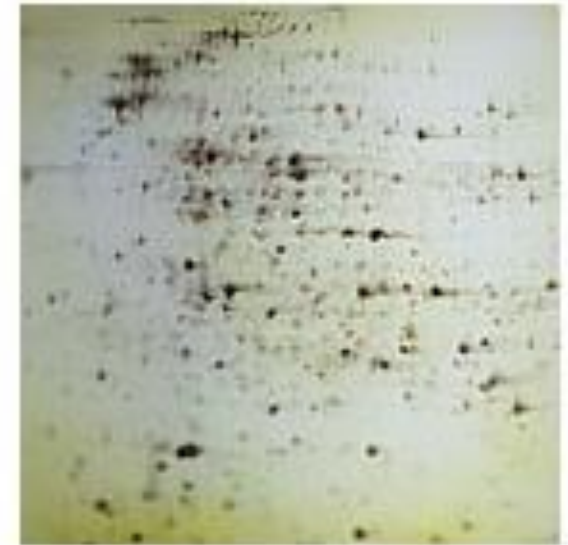
Sypro Ruby

1.4 ng



Coomassie

36 ng



silver

0.6 ng

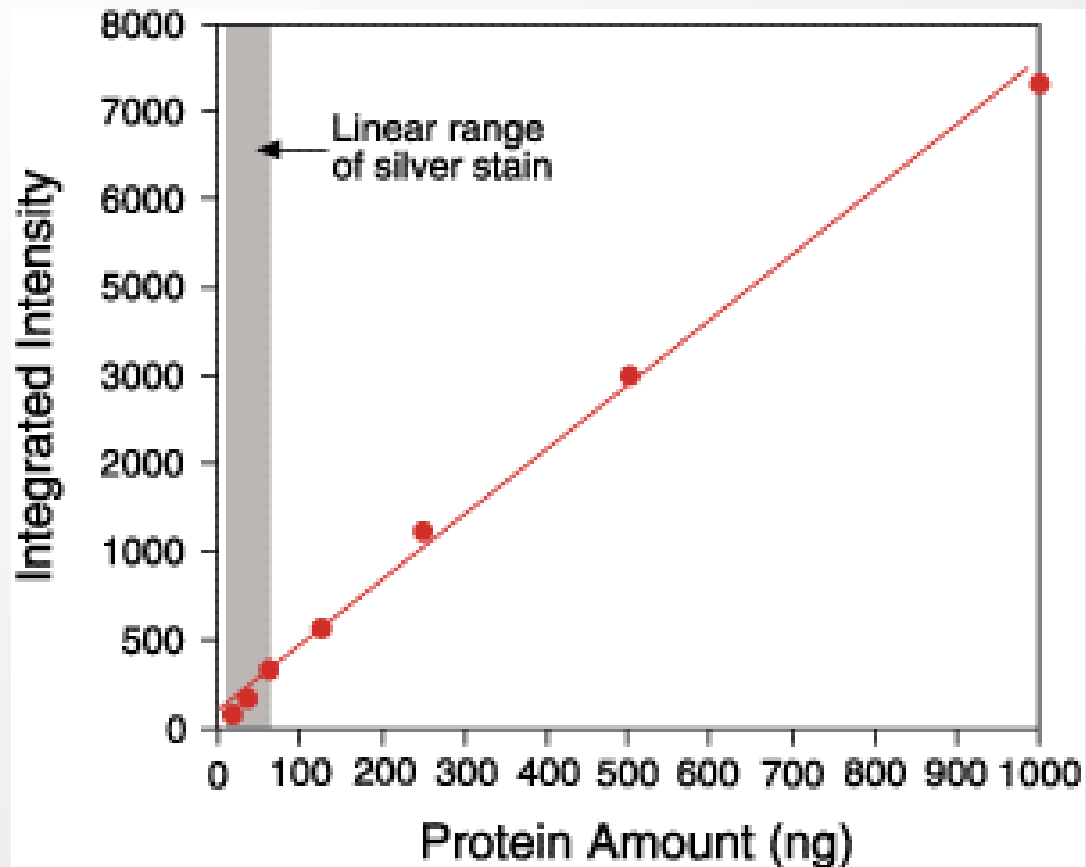
PTM specific staining

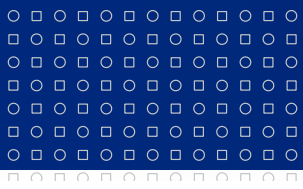
Pro-Q Diamond

Pro-Q Emerald

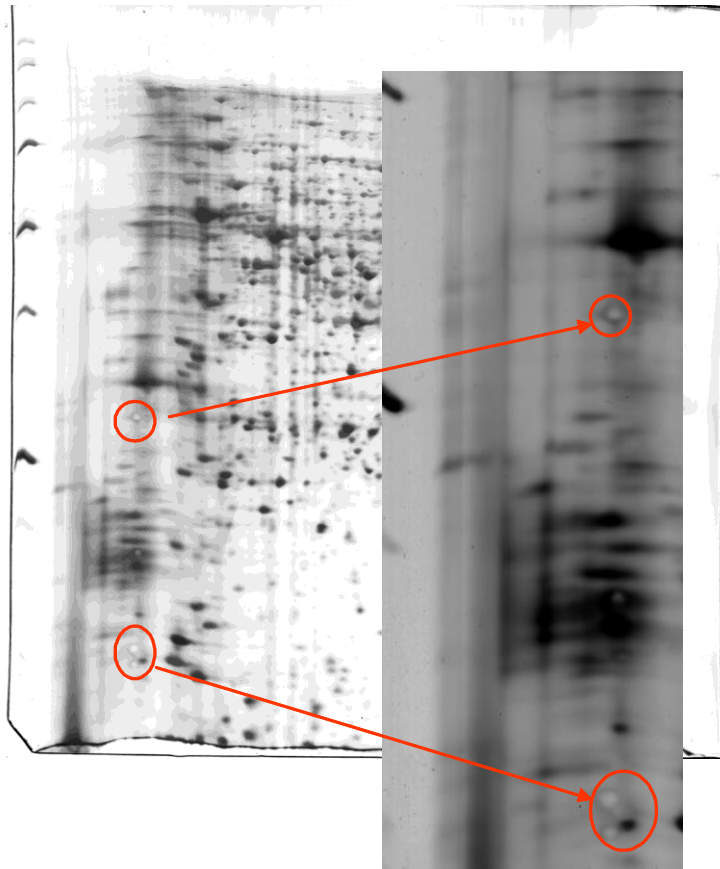
PROTEIN STAINING – LINEARITY

Sypro Ruby





Ag



Sypro Ruby

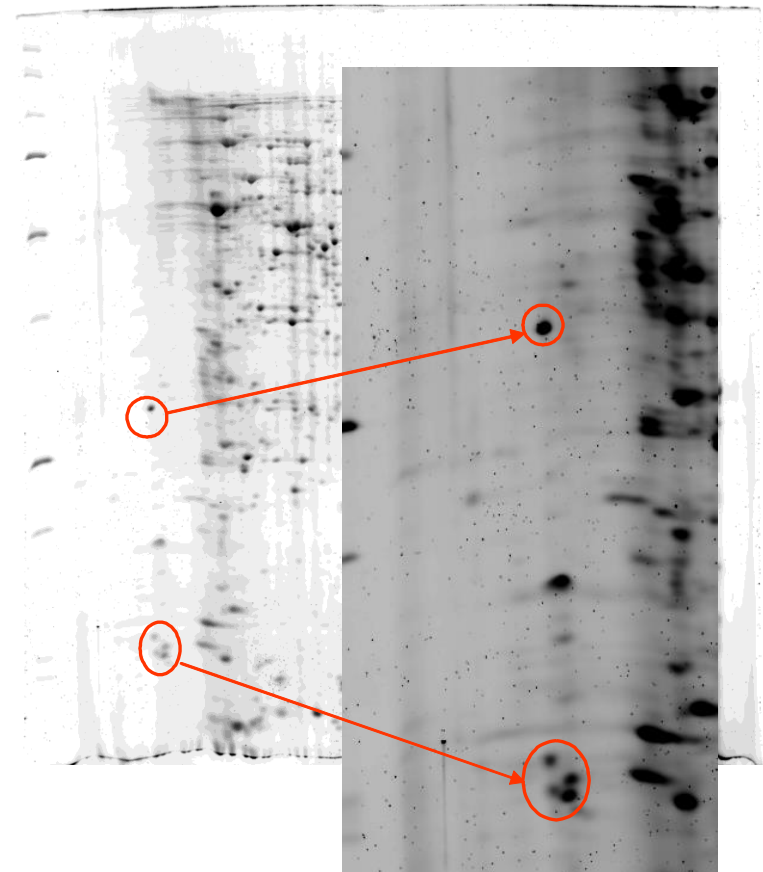
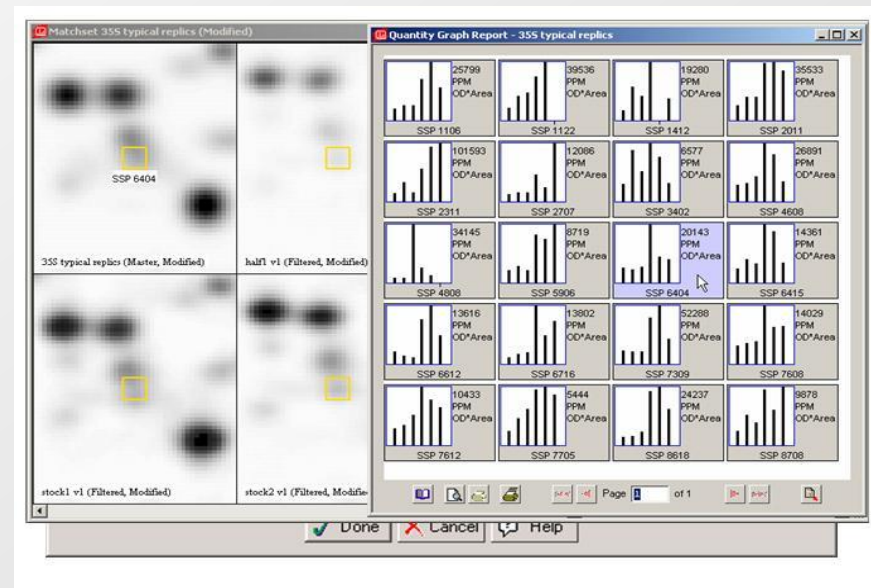
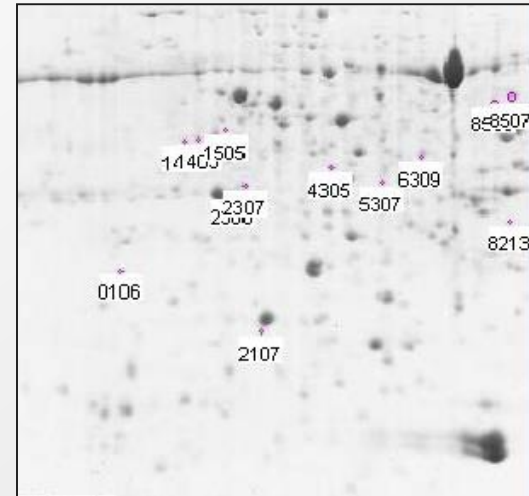
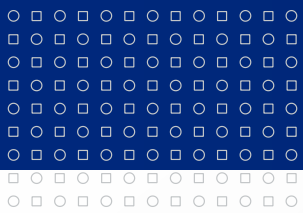


IMAGE ANALYSIS

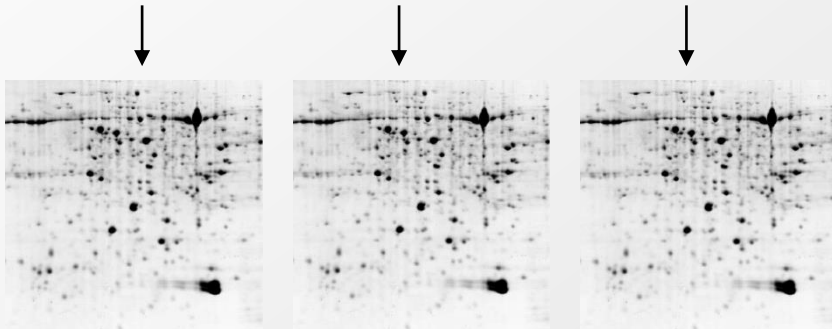
- quality
- quantity





biological variability

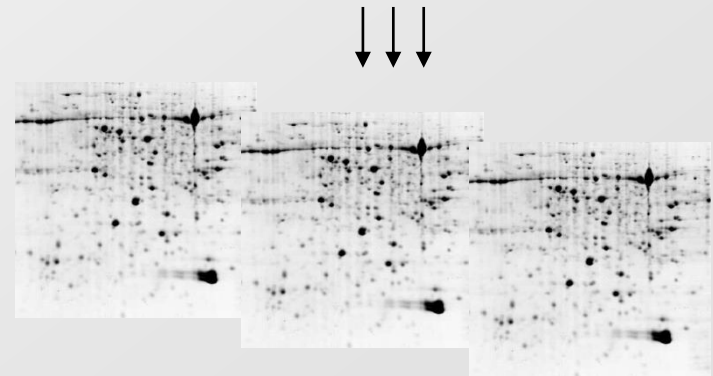
three plantelets analysed separately under same conditions



biological replicates

technical variability

same plantelet analysed three times under same conditions



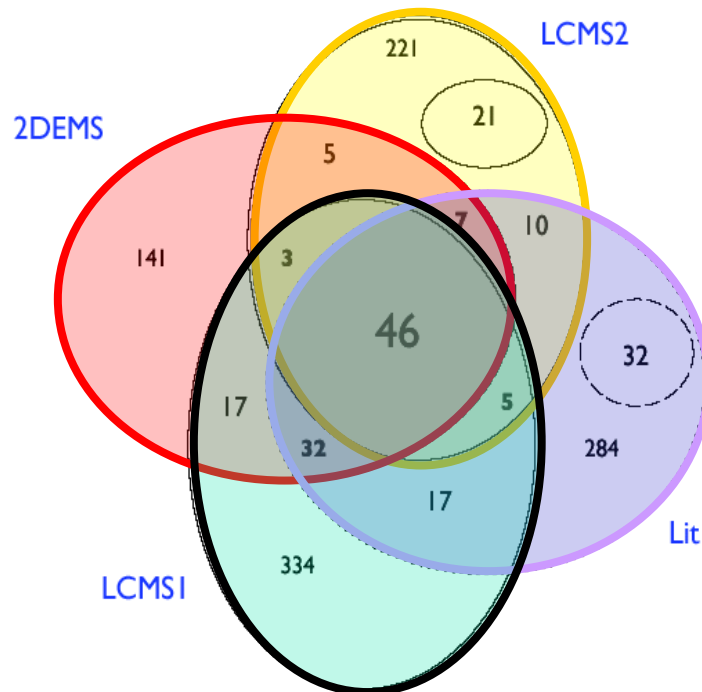
technical replicates



2D or not 2D ?

- visual aspects
- reproducibility
- dynamic range
- extreme proteins (membrane, basic...)
- difficult automatization
- postdigestion extraction

Different Platforms See Different Plasma Proteomes: Small Overlap of Four Plasma Proteome Datasets (Number of NR proteins)



- **46** proteins in all four lists
- 195 proteins in 2 or more lists
- **1175** NR proteins total

MULTIDIMENSIONAL CHROMATOGRAPHY

FOR

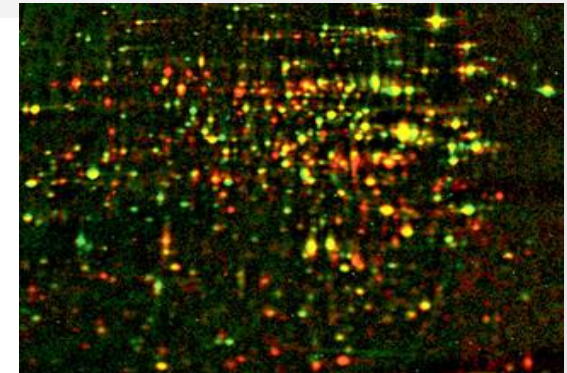
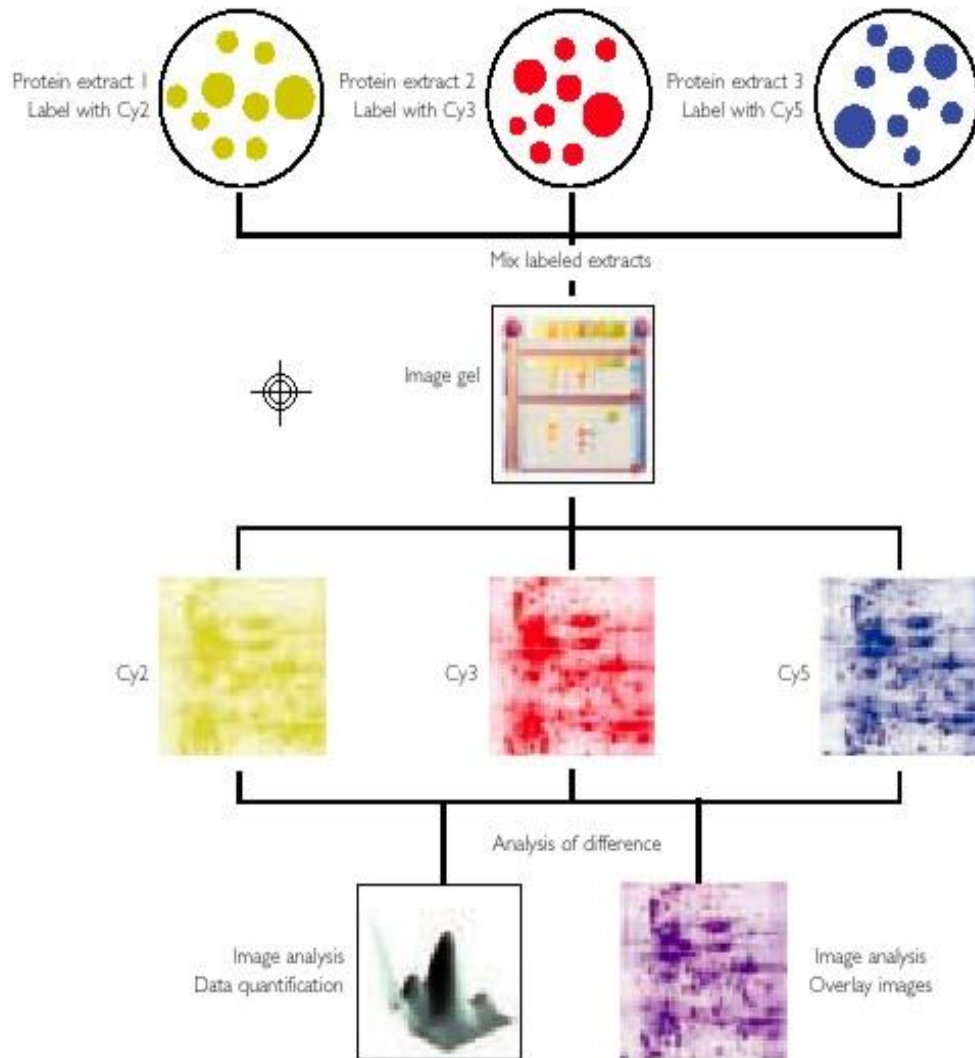
- large sample volumes
- on-column concentration
- membrane proteins, basic proteins
- no staining
- peptides – going directly to MS
- automatization

AGAINST

- vizual aspects lost: pI a M_r
- LC - serial analysis
- GE - more samples in paralel

Difference Gel Electrophoresis

DIGE



BIOMARKERS

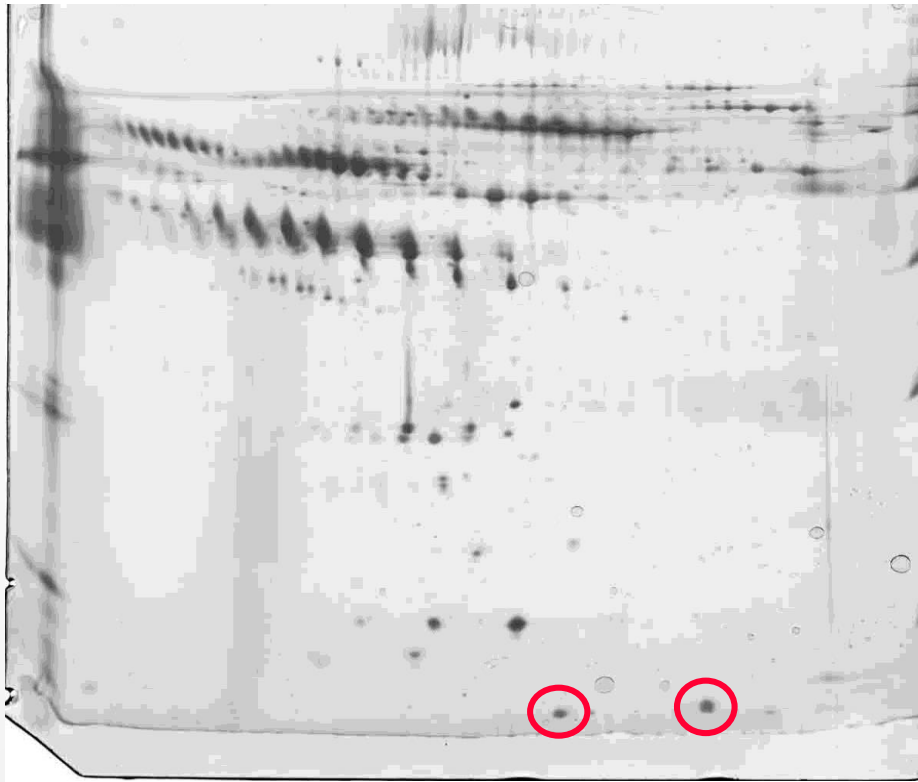
... NEEDLE IN HAYSTACKS

prefractionation ▪ separation ▪ identification ▪ control vs. sample

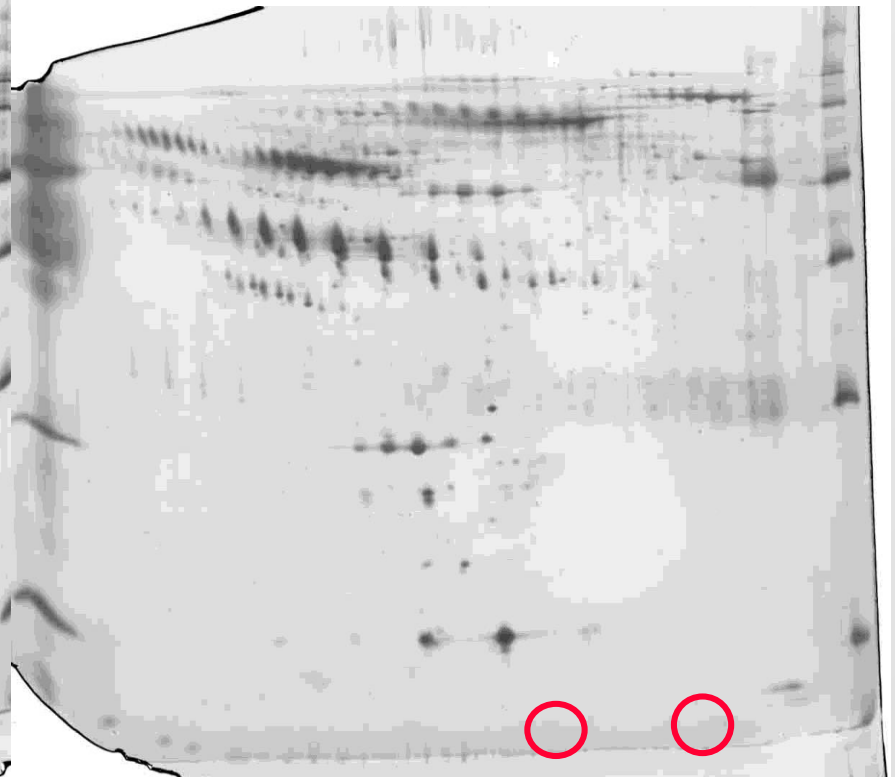
- **haystack** - proteins without relation to disease
- **needles** – disease specific proteins
- potential needles **difficult to validate** **biological variability!**
- are needles worth further examination?
- often contain **PTM**, difficult to be identified by MS

Biomarkers in human plasma

Day 21 – before clinical manifestation



Day 44 – after clinical manifestation



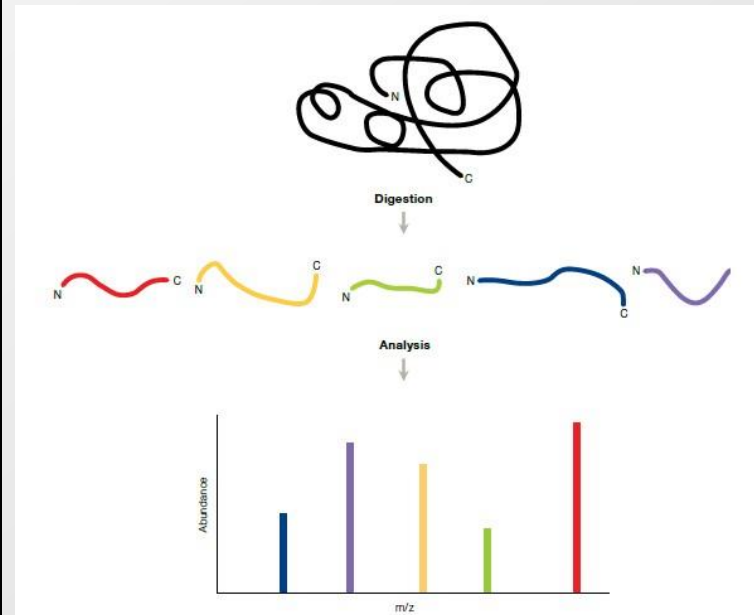
separation → identification

↓ **DIGEST**

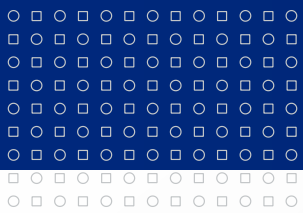
trypsin Glu-C Asp-N thermolysin

MAVEPFRRPITRPHASIEVDTSGTGGSAGSSEK**VF**
CLIGQAEGGEPNTVYELRNYAQAKRLFR**SGEL**LD**AI**
ELAWGSNPNYTAGRILAMRIEDAKPASAEIGGLKIT
 SKIYGNVANNIQVGLEKNTLSDSLRLRVIFQDDRFN
EVYDNIGNIFTIKYKGEEANATFSVEHDEETQKASR
 LVLKVG**DQEVKSYDLTGGAYDYTNAIITDINQLPDF**
 EAKLSPFGDKNLESSKLDKIENANIKDKAVYVKAVF
 GDLEKQTAYNGIVSFEQLNAEGEVPSNVEVEAGEE
 SATVTATSPIK**TIEPFELTKLKGGTNGEPPATWADKL**
DKFAHEGGYYIVPLSSKQSVHAEVASFVKERSDAGE
PMRAIVGGGFNESKEQLFGRQASLSNPRVSLVANS
GTFVMDDGRKNHVPAYMVAVALGGLASGLEIGES
 ITFKPLRVSSLDQIYESIDLDELNENGIISIEFVRNRTN
TFFRIVDDVTTFNDKSDPVKAEMAVGEANDFLVSE
LKVQLEDQFIGTRTINTSASIIKDFIQSYLGRKKRDN
EIQDFPAEDVQVIVEGNEARISMTVYPIRSFKKISVS
 LVYKQQT**LQA**

- IN-GEL
- IN-SOLUTION



MS



G I G O



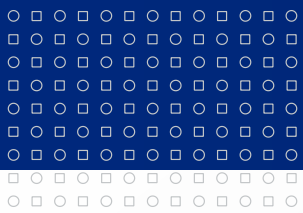
G I G O

GARBAGE IN - GARBAGE OUT



LITERATURE

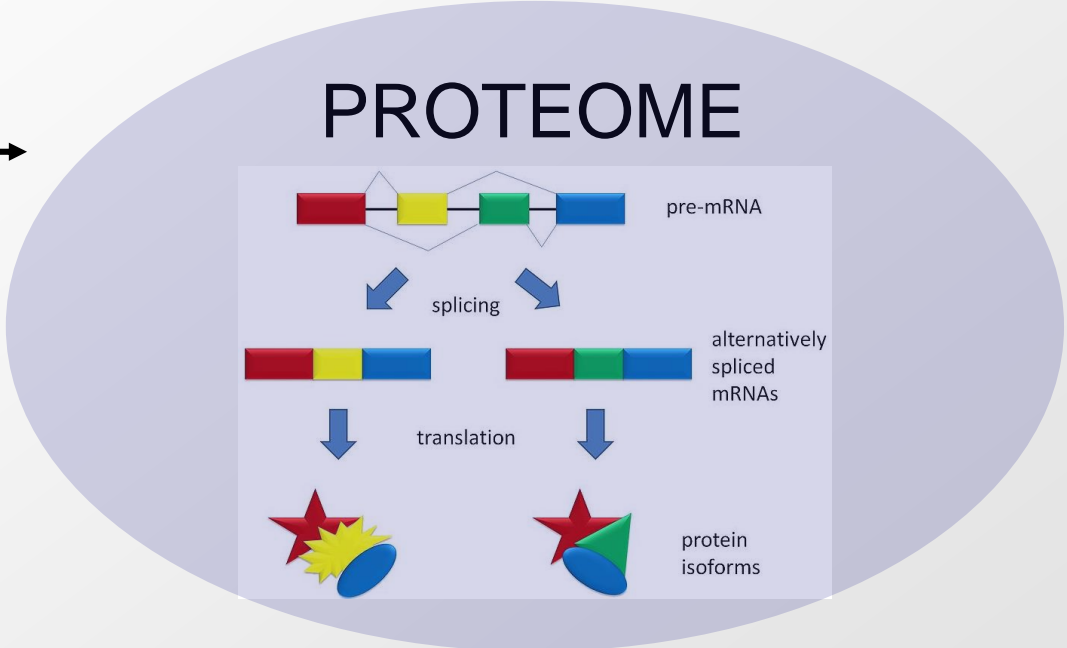
- R.M. Twyman: Principles of Proteomics
- R.Westermeier, T.Naven, H-R Höpker: Proteomics in Practice
- A.J.Link: 2D Proteome Analysis Protocols
- Current Protocols in Protein Science
- R.J.Simpson: Proteins and Proteomics
- T.Rabilloud: Proteome Research: Two-dimensional Gel Electrophoresis and Identification Methods
- A. Görg, W. Weiss, M.J.Dunn: Proteomics 2004, 4, 3665, rev.
- I. Miller, J. Crawford, E. Gianazza: Proteomics 2006, 6, rev.
- F.Chevalier: Proteome Science 2010, 8:23, review
- R. Burgess, M. Deutscher: Guide to Protein Purification



I. SEPARATION
II. PREFRACTIONATION



GENOME



ISOFORMS

PTM ~200 variants (fosforylation, glykosylation, acylation, methylation...)

CONCENTRATION RANGE ~ 10 orders of magnitude



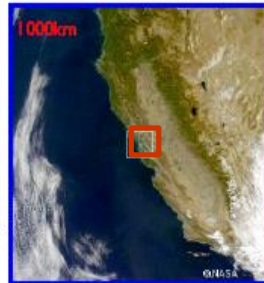
PREFRACTIONATION → **MS**



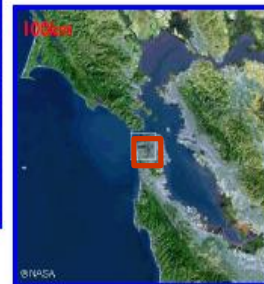
10^{10} Really Is Wide Dynamic Range



10 10 000km



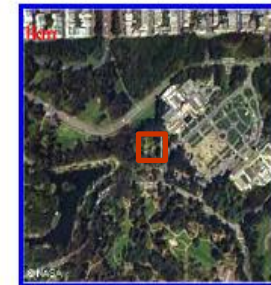
9 1 000km



8 100km



7 10km



6 1km



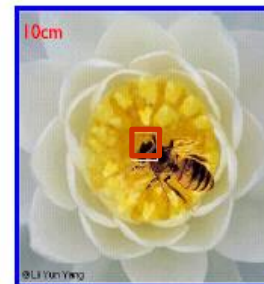
5 100m



4 10m



3 1m

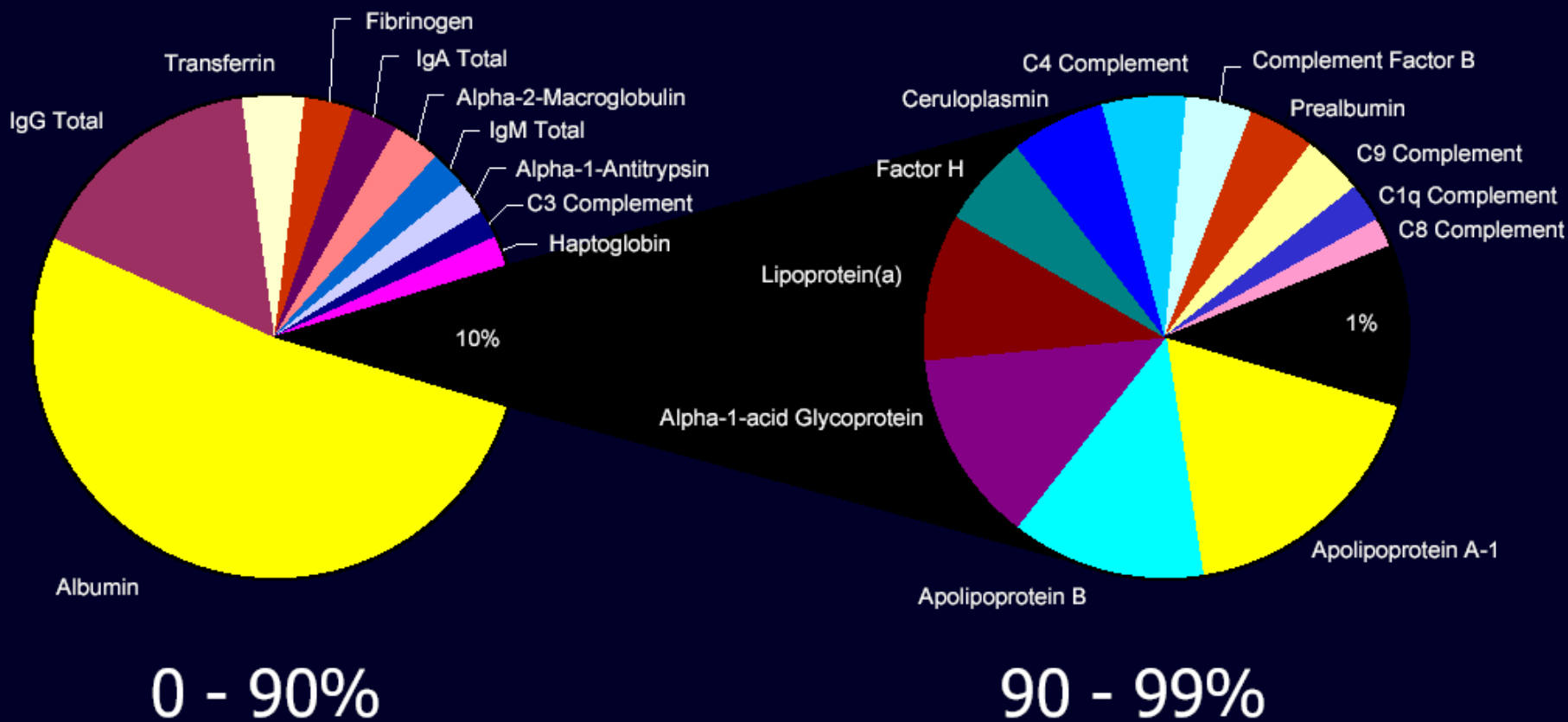


2 10cm



1 1cm

Abundant proteins in human plasma

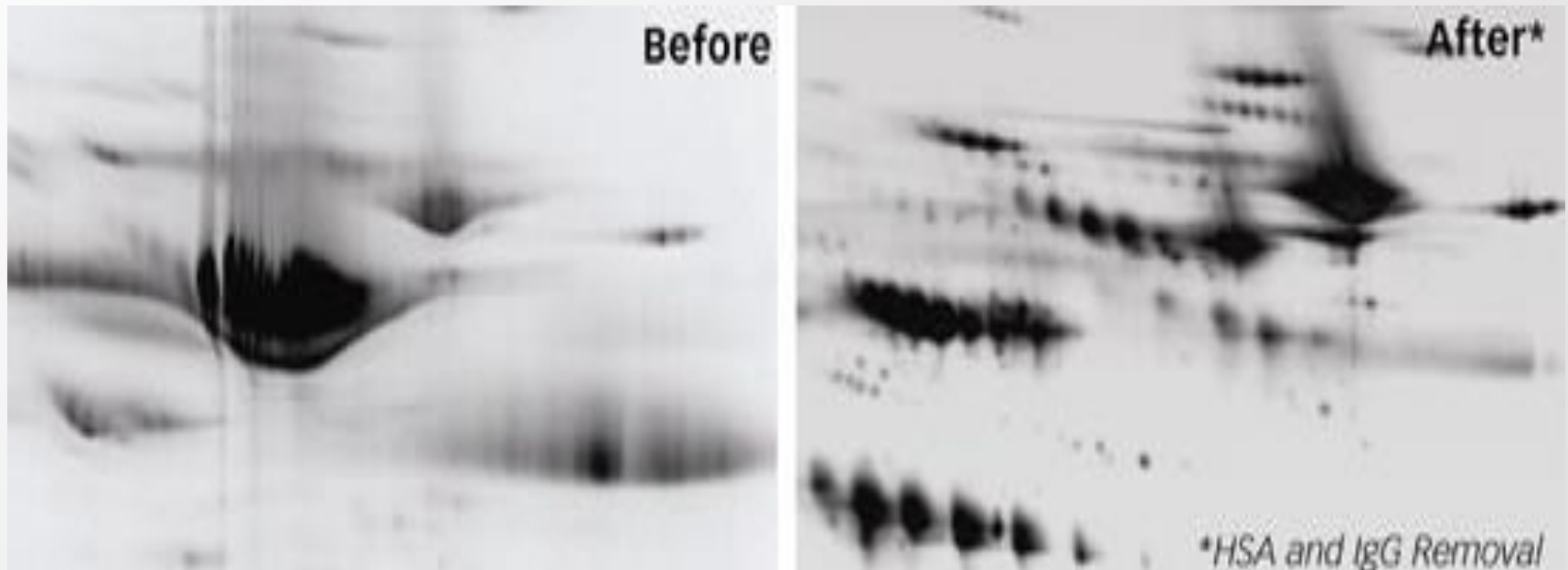


AFFINITY DEPLETION

Removal of abundant proteins by affinity chromatography

HSA

IgG



Human plasma – bound fractions after affinity depletion

ALBUMIN

IgG

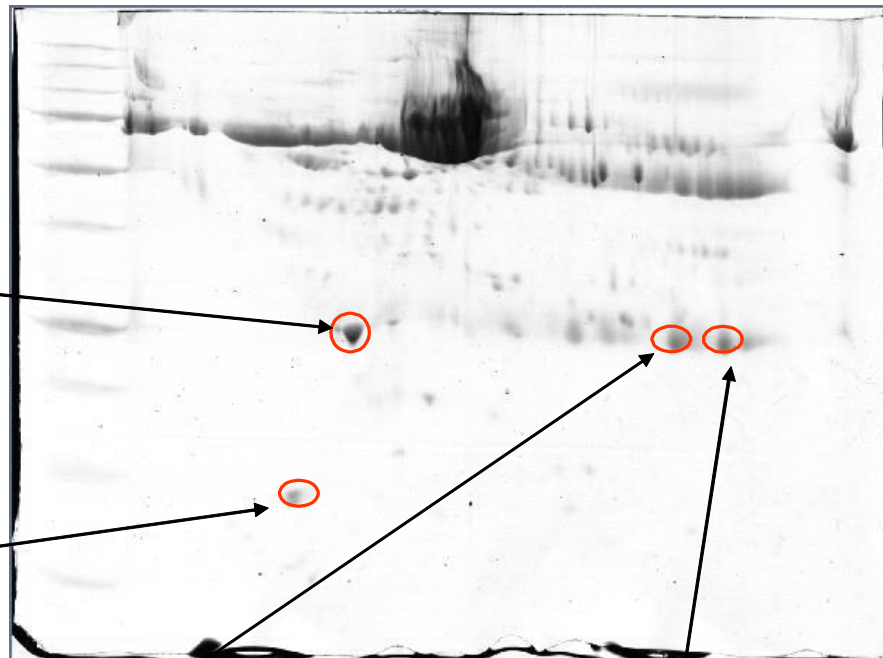
Staining CBB G-250

Apolipoprotein

albumin

Immunoglobulin kappa light chain

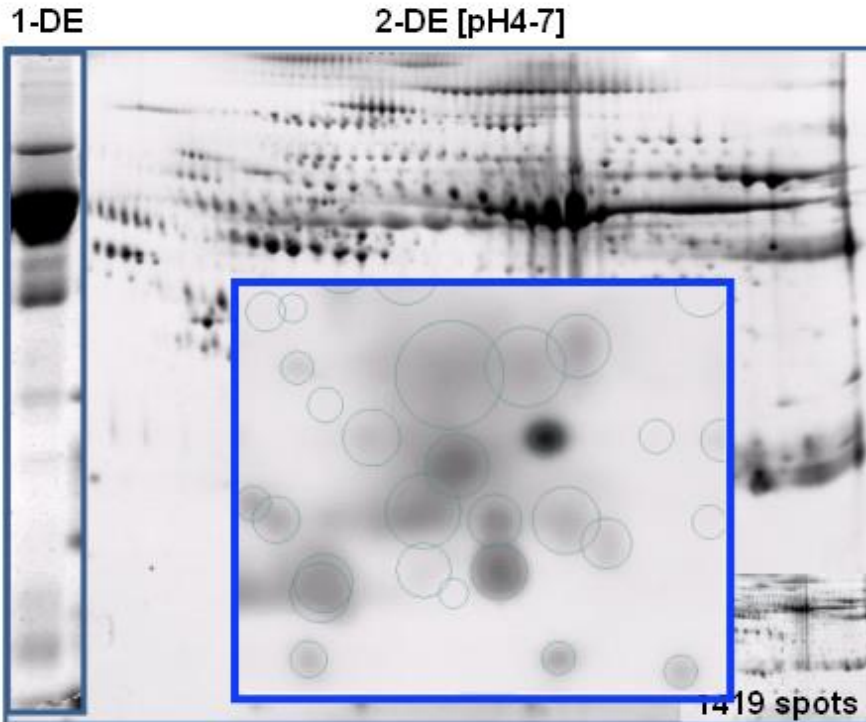
Immunoglobulin light chain



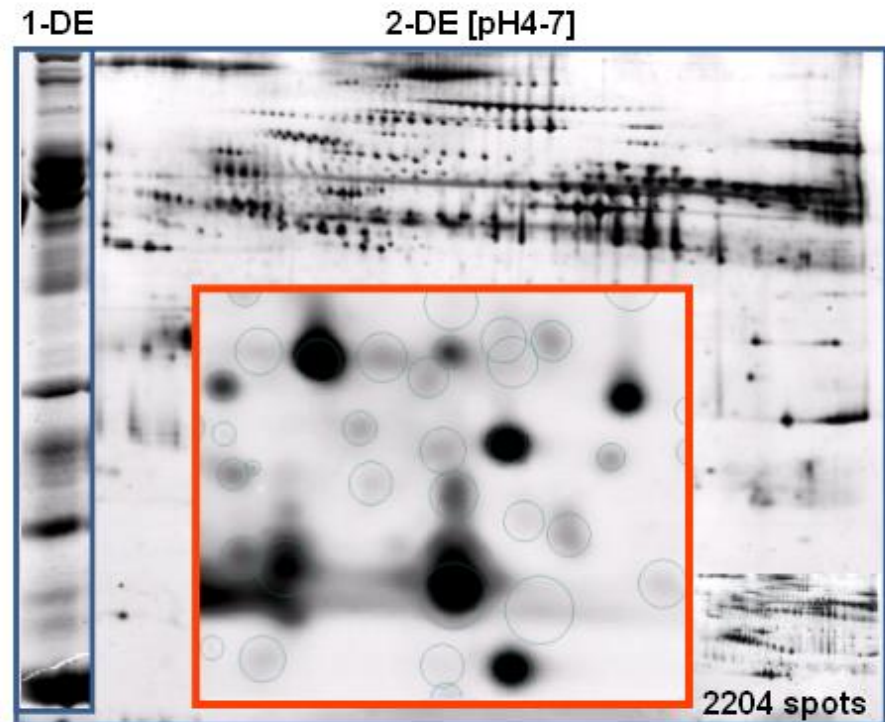
CPPL Combinatorial Peptide Ligand Library



Native Human Serum

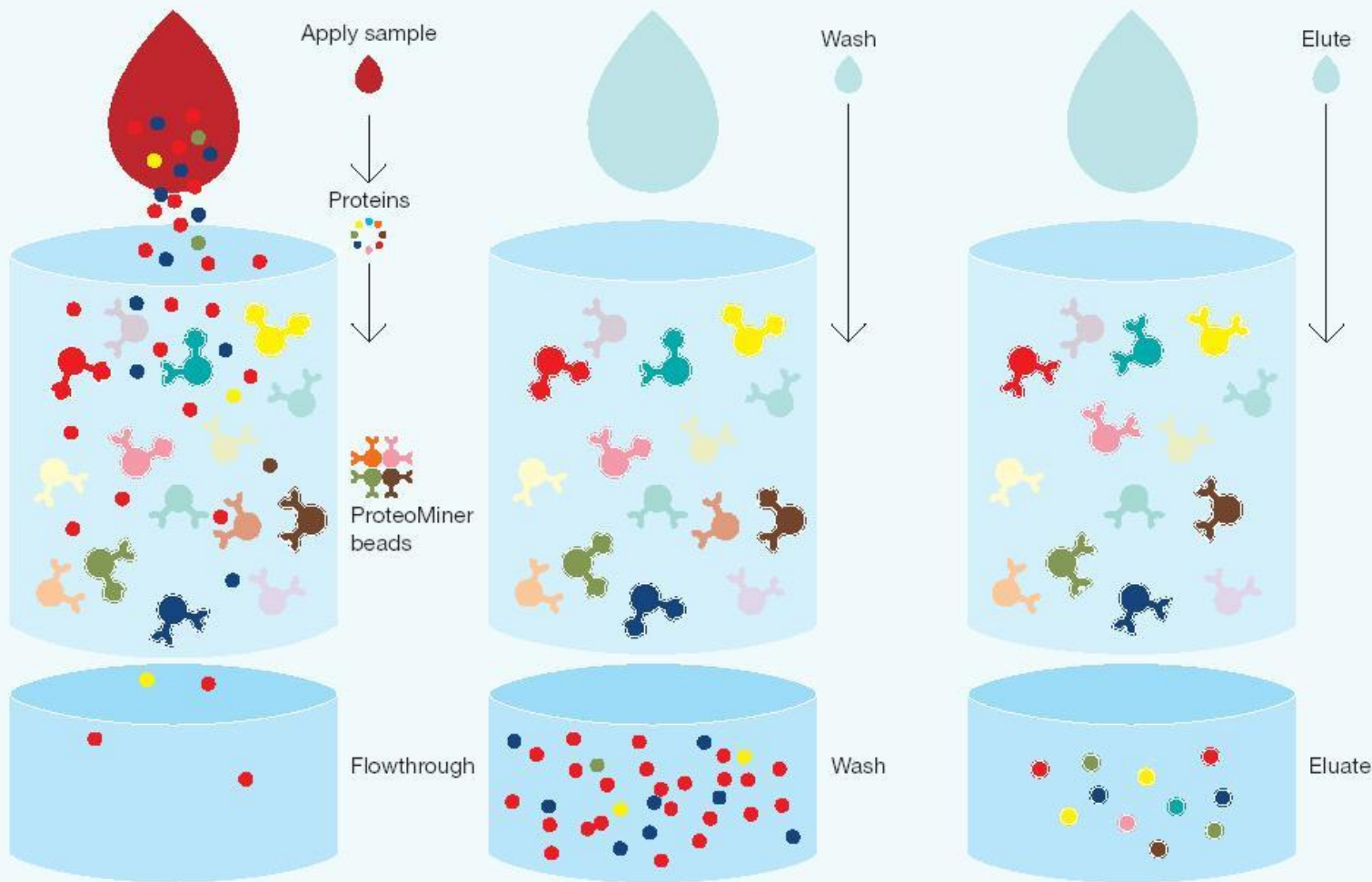


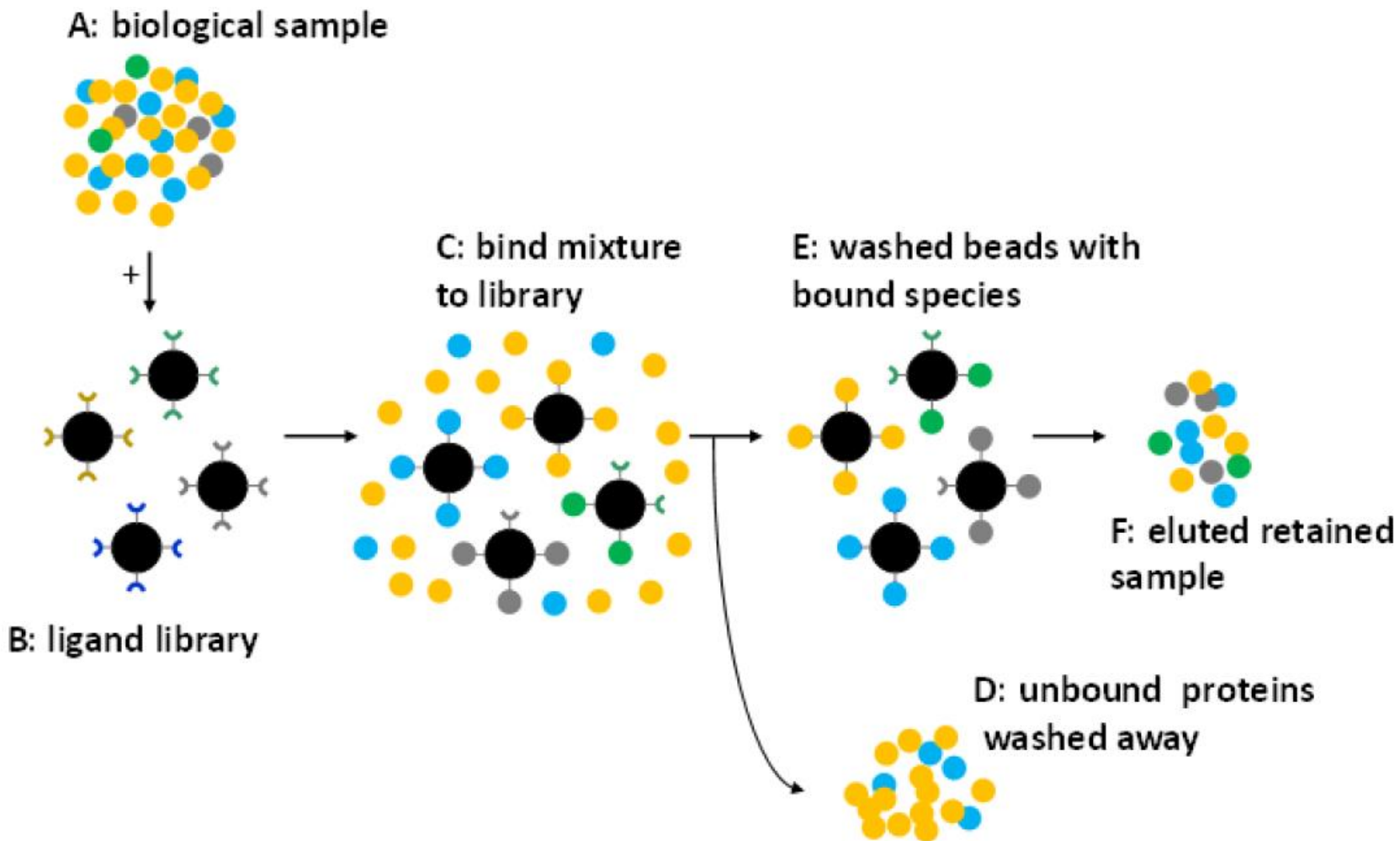
Human Serum Fractionated by ProteoMiner

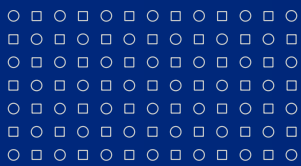


PREFRACTIONATION

PROTEOMINER







IEF prefractionation



MicroRotor

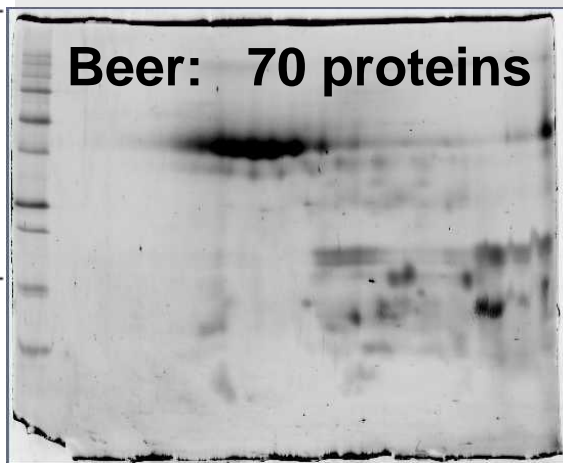
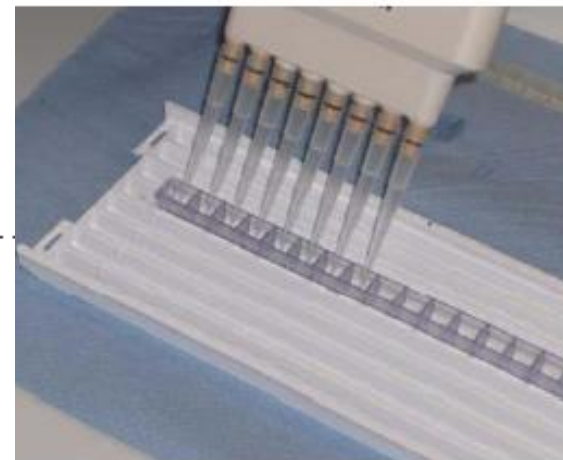
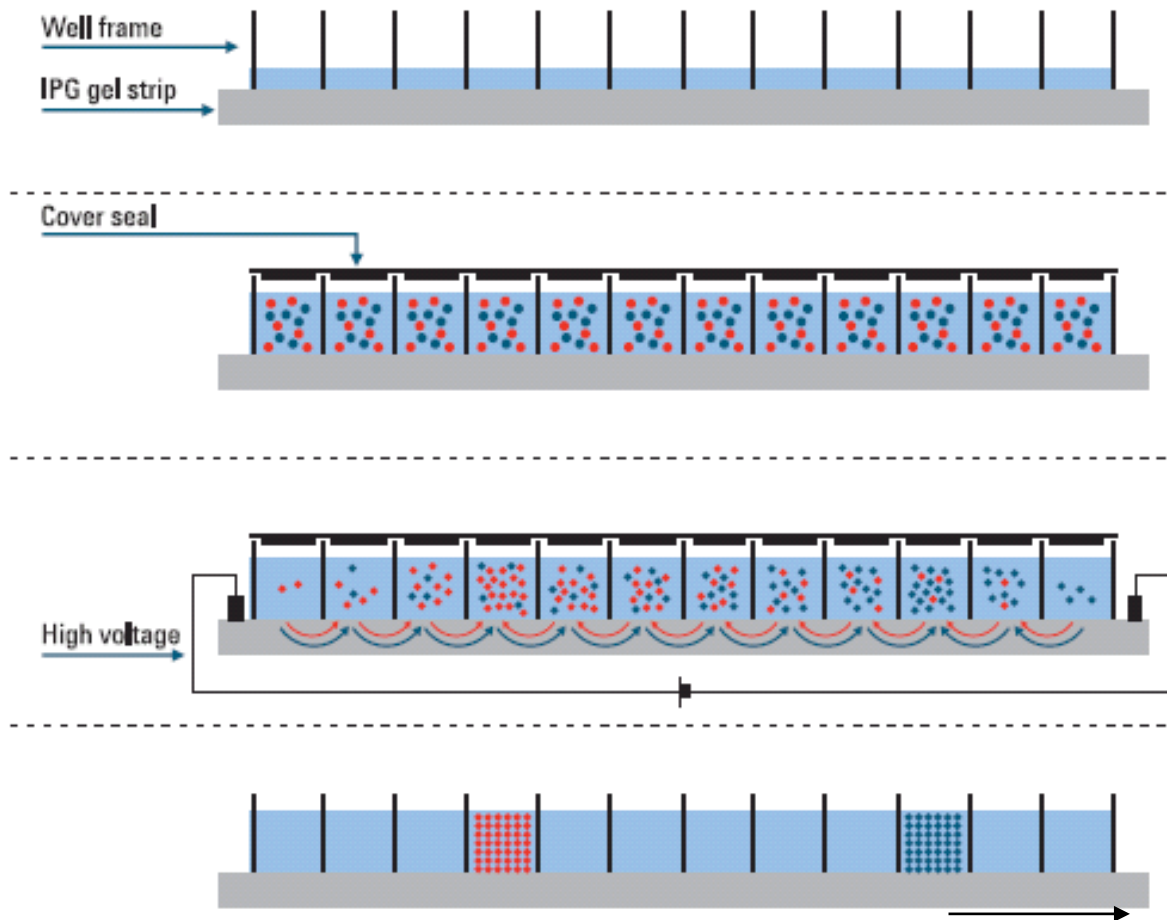
- prefractionation in solution



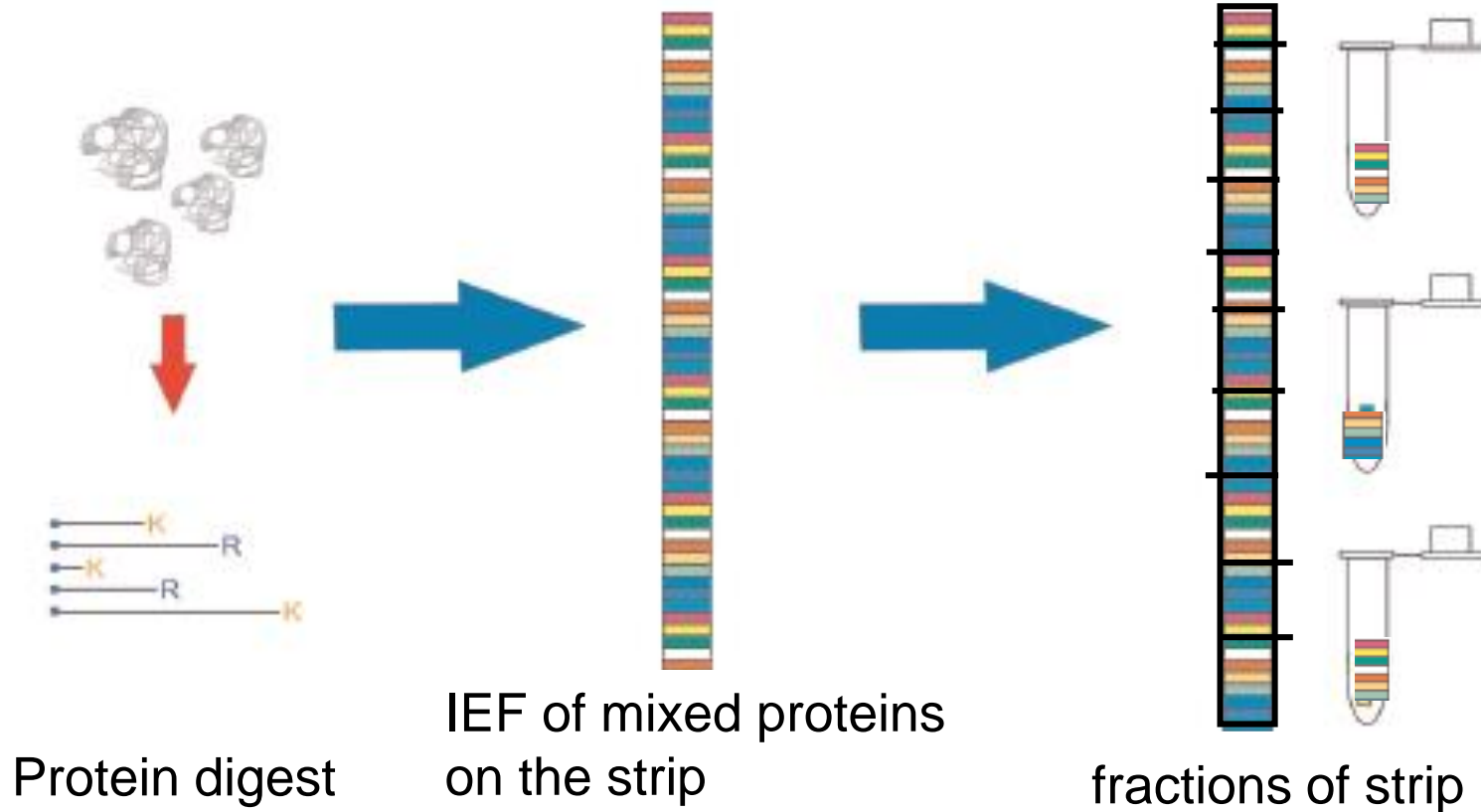
OffGel Fractionator

- prefractionation in solution using IPG strip

OFFGEL IEF prefractionation of proteins or peptides

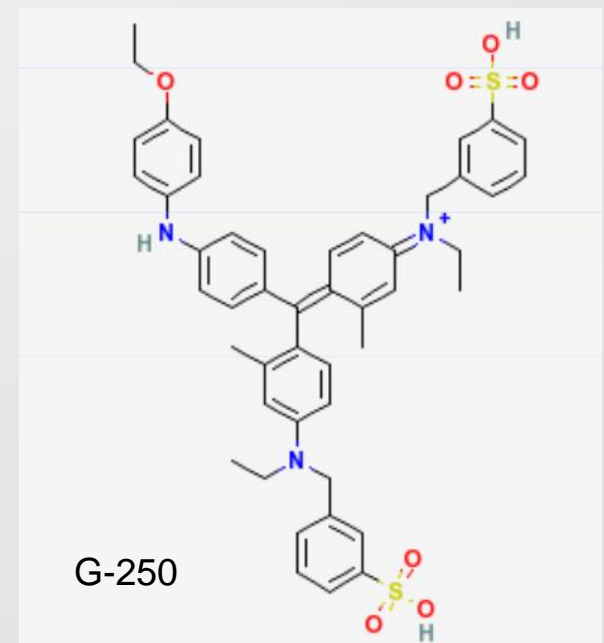
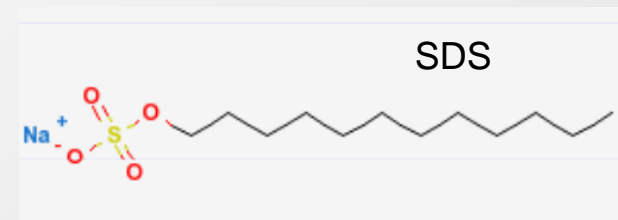
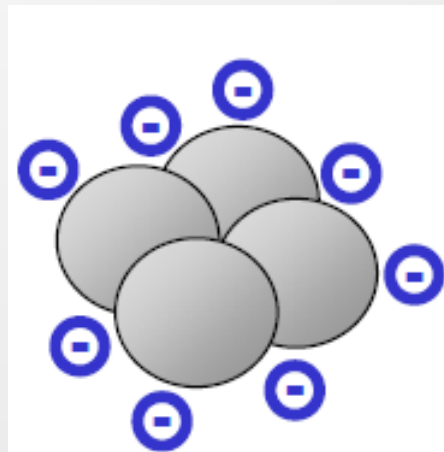


IPG-IEF



Blue Native Electrophoresis BNE

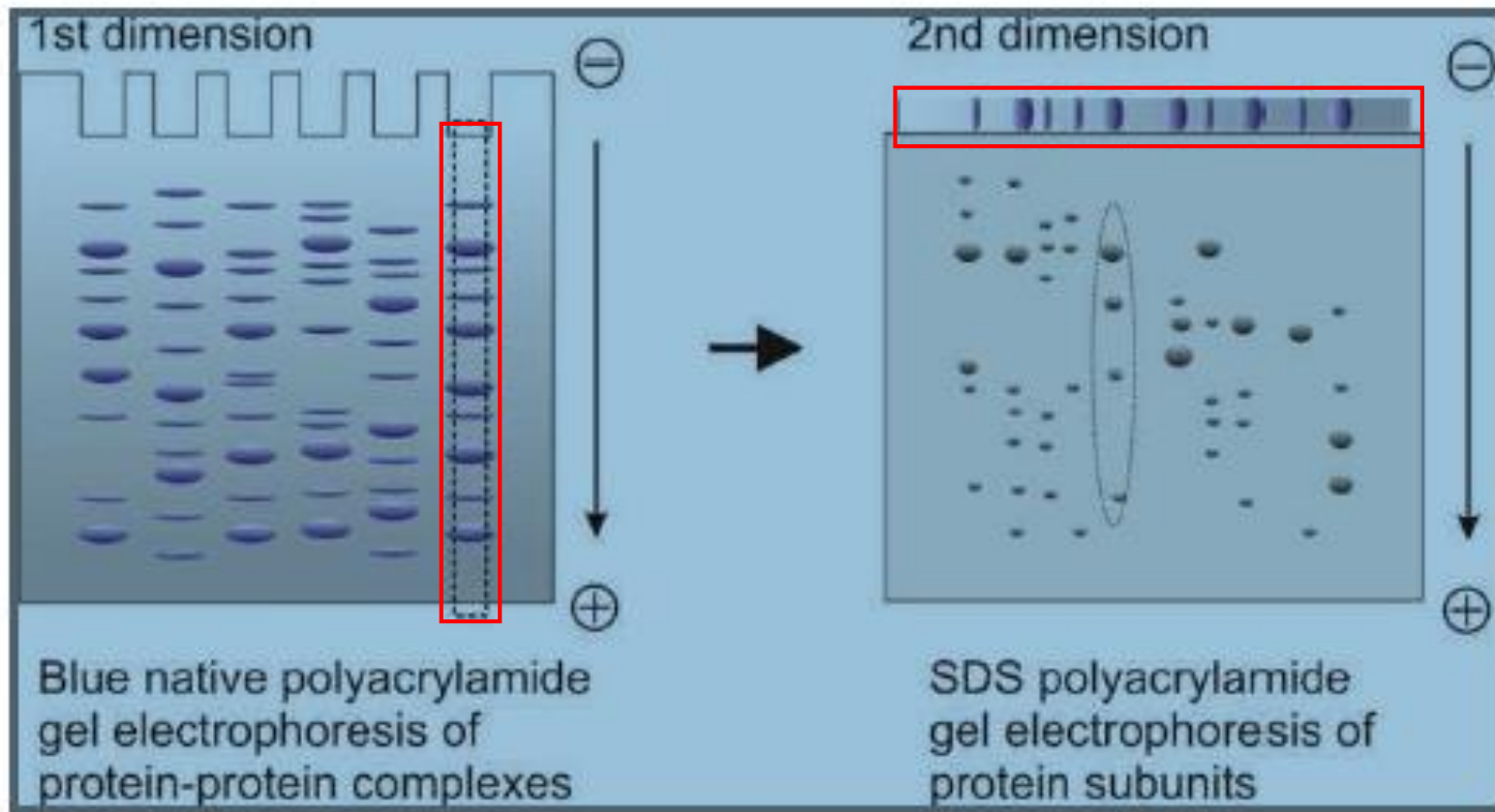
- Separation of native proteins
- Separation of membrane complexes
- Solubilization by non-ionic detergents
- Charged by **Coomassie G-250**
- BN PAGE gel (strip/band) as 2D

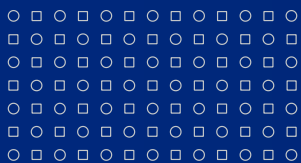


2DE

BNE

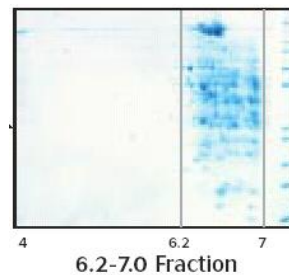
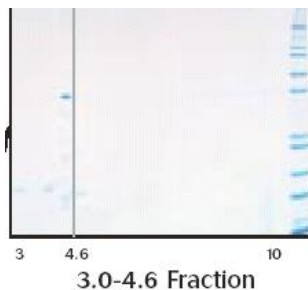
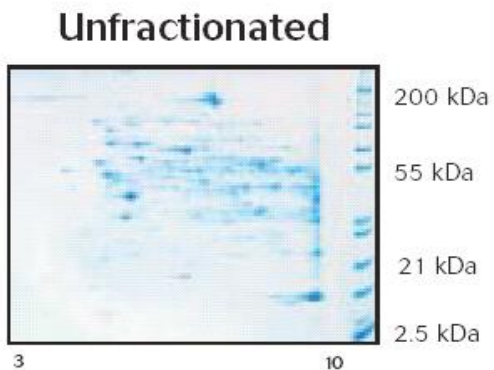
SDS-PAGE



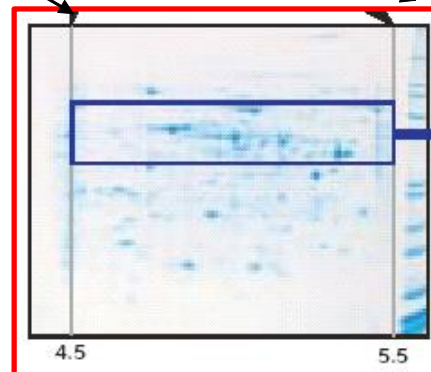
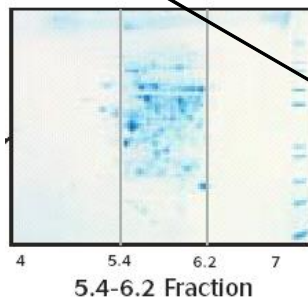
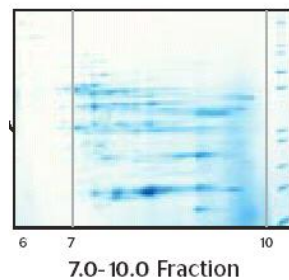
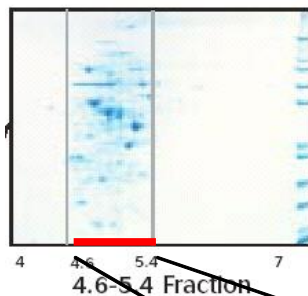


PREFRACTIONATION

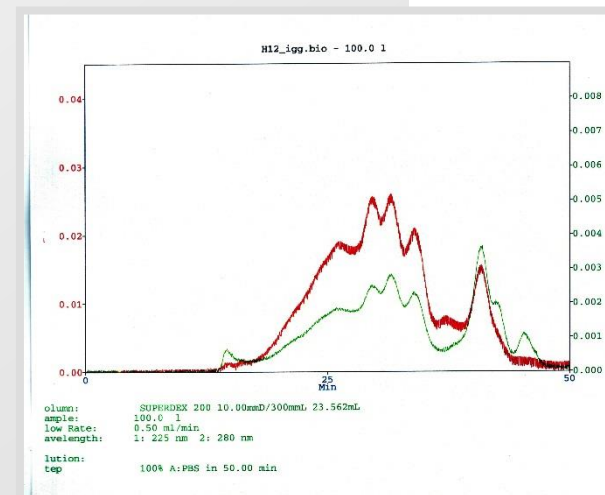
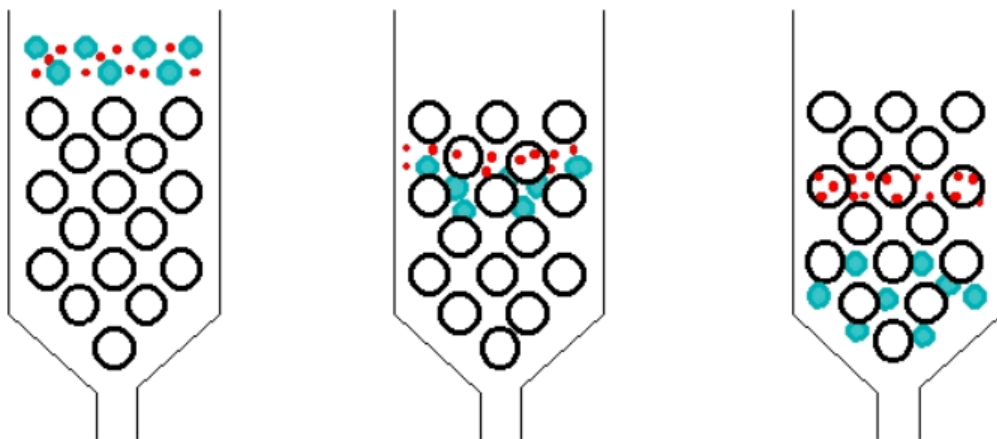
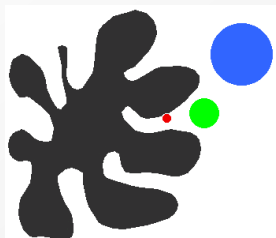
MICRO RANGES



pl



GEL CHROMATOGRAPHY



FASP Filter aided sample preparation

PROTEINS



Lysate
SDS DTT

8M Urea
IAA

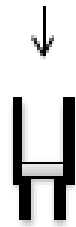
Ammonium
bicarbonate

Trypsin

proteins
nucleic
acids



spin



spin



spin



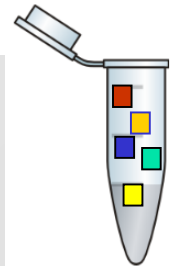
incubation
spin



↓
SDS
DTT

↓
8M Urea
IAA

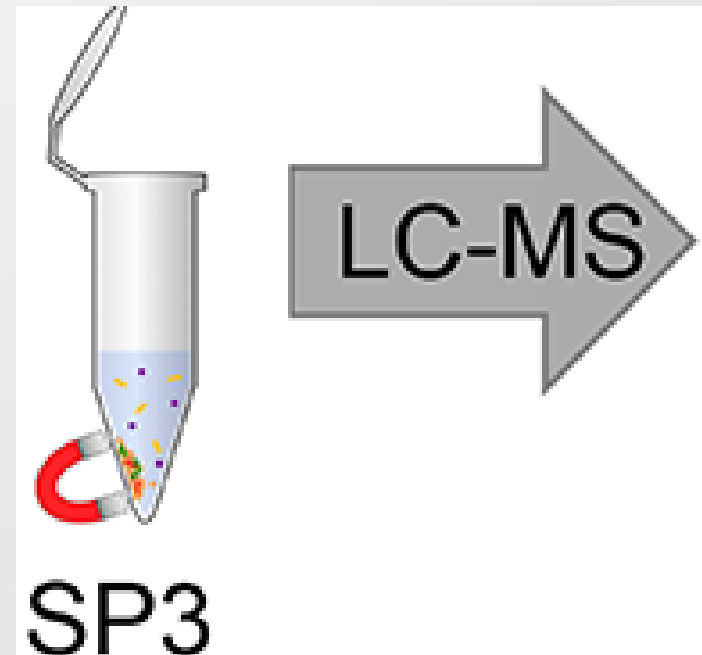
PEPTIDES



“single vessel” approach:

SP3 single-pot solid-phase-enhanced sample preparation

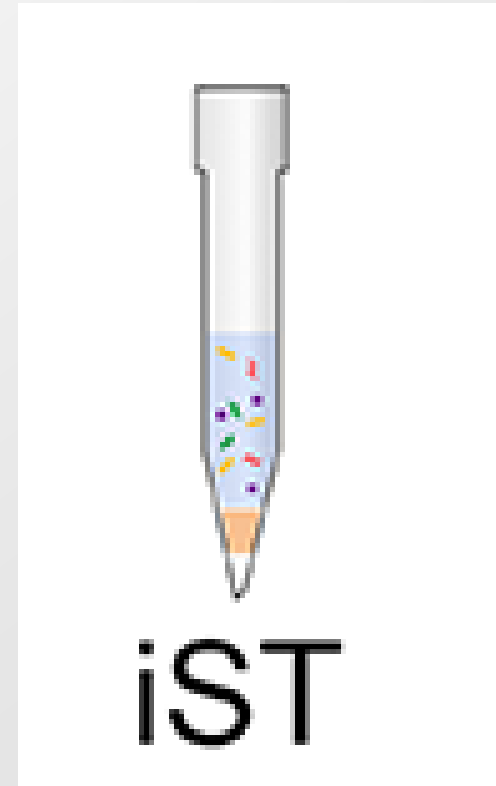
- surface-functionalized (i.e. carboxylate-coated) paramagnetic beads trap proteins and peptides in hydrophilic layers when the organic composition of the buffer is increased and the pH adjusted.
- the beads can be immobilized within a magnetic field
- efficient removal of contaminating agents including chaotropes and detergents by washing with different organic solvents (i.e., ethanol and acetonitrile)
- after rinsing, bound proteins or peptides can be eluted from the beads using an aqueous solution.
- **protein cleanup, enzymatic digestion, desalting, and peptide recovery in a single tube.**



“single vessel” approach

iST in-StageTip method

- complete sample preparation in a single reactor
- resembles an in-solution digestion with the advantages of a single FASP-like reaction vessel that avoids the use of a filter membrane.
- the C18 disk serves as a physical barrier for insoluble material and macromolecules.
- additionally, it enables final peptide cleanup using solid-phase extraction (SPE).
- One drawback of iST as compared to FASP is the limitation regarding the use of certain reagents (i.e., iST cannot remove SDS).



MOTIVATING LITERATURE FOR ADVANCED READERS

Two-dimensional gel electrophoresis in proteomics: A tutorial

Thierry Rabilloud et al. *Journal of Proteomics* 2011

Two-dimensional gel electrophoresis in proteomics: past, present and future

Thierry Rabilloud et al. *Journal of Proteomics* 2010

Proteomic biomarker discovery: It's more than just mass spectrometry

Josip Blonder et al. *Electrophoresis* 2011

Basics and recent advances of two dimensional – polyacrylamide gel electrophoresis

Sameh Magdeldin et al. *Clinical Proteomics* 2014

Evaluation of FASP, SP3, and iST Protocols for Proteomic Sample Preparation in the Low Microgram Range

Malte Sielaff et al. *J. Proteome Res.* 2017

For all the complex problems and difficult questions
there is always one simple, easily comprehensible
w r o n g answer.

